If sports supplements and ergogenic aids work, they're probably dangerous and/or banned

Products that claim to be performance enhancing are often used by athletes. Many of these drugs and supplements have not been proven conclusively to have an ergogenic effect. Steroids and recombinant erythropoietin are ergogenic, but they are associated with serious adverse events and their use is banned by many sporting associations. Steroid precursors and pyruvate are not ergogenic. Variable results have been obtained with β-hydroxy-β-methylbutyrate. Over-the-counter products claiming to contain human growth hormone and insulin-like growth factor-1 have not been proven to be performance enhancing. In athletes with a well balanced diet, protein, amino acid and antioxidant supplementation are generally unnecessary. Caffeine, creatine, ephedrine and pseudoephedrine are ergogenic in some, but not all, sporting activities.

Little proof for product claims

Many products (see table 1) claim to be performance enhancing and are popular with recreational and elite athletes. However, there is often a lack of objective evidence to support claims of an ergogenic effect with these drugs and supplements.[1] Performance-enhancing products are often purchased based on popular magazine advertisements or peer or coach recommendations rather than professional medical advice. Most major sport governing bodies prohibit the use of drugs that are used for medical purposes, but that also have ergogenic properties.[1]

Where it all began: anabolic steroids

The athletic world's fascination with ergogenic aids started with anabolic steroids (e.g. dianabol, nandrolone, stanozolol).[1,2] These agents, which are available in oral and injectable forms, are synthetic derivatives of testosterone.

They are ergogenic, but banned ...

Anabolic steroids increase muscle protein synthesis and, therefore, increase muscle mass.[2-4] Some evidence also suggests that anabolic steroids increase erythropoiesis.[3] The International Olympic Committee (IOC) bans the use of anabolic steroids; the most common test measures the ratio of testosterone to its metabolite, epitestosterone (T : E ratio).[1] This ratio is 1 : 1 in most men; the IOC allows a generous ratio of 6 : 1.[1]

... with established adverse effects

The adverse consequences of using anabolic steroids are well established (table 1).[1-3] In addition, there are psychosocial aspects of anabolic steroid use; significant associations have been found between anabolic steroid use and low school achievement, low self-esteem and the use of prescription sedatives.[1]

Steroid precursors not ergogenic ...

The most popular over-the-counter (OTC) androgenic precursors are androstenedione and dehydroepiandrosterone (DHEA) [table 1].[1] These agents are classified as dietary supplements in the US, which means the product claims do not have to be verified by the US FDA.

These steroid precursors do not promote muscle protein synthesis.[1] For example, men receiving androstenedione[5] or DHEA[6] while participating in a 12-[5] or 8-week[6] resistance-training programme experienced similar changes in body composition and strength to those receiving placebo.

May cause 'blurring of the sexes'...

Reported irreversible effects of DHEA include gynecomastia in men (possibly as a result of elevated estrogen levels) and virilisation, including hair loss, hirsutism and voice deepening, in women.[7] DHEA users may also be at increased risk of developing uterine or prostate cancer.[7]

In male androstenedione recipients, estradiol levels increased by 97% after 12 weeks.[5] In the same study, androstenedione recipients experienced a reduction from baseline in mean high-density lipoprotein cholesterol (HDL-C) and an increased cardiac lipid profile risk, while placebo recipients experienced an increase in HDL-C and a decrease in cardiac lipid profile risk.[5]

... and you'll lose your gold medal

Both androstenedione and DHEA are banned by a number of sporting associations, including the IOC.[1] Use of these substances can alter the T : E ratio, resulting in a positive drug test for testosterone.[1]

Moreover, the description of the contents of these products is often misleading. In two US studies, the

Table 1. Popular sports supplements and ergogenic aids

Product	Ergogenic potential	Adverse effects/safety	Typical dosage	Comments
Anabolic steroids	Yes	Hirsutism, acne, menstrual irregularities, aggression, ↓ spermatozoa, ↑ cardiovascular risk, liver dysfunction, closure of growth plates	250–3200 mg/wk, PO or injectable. Many dosage strategies exist	Enhances protein/muscle synthesis
Antioxidants	No	None known	Megadoses of ascorbic acid (vitamin C), tocopherol (vitamin E), β-carotene, etc.	Although not ergogenic, a diet high in antioxidants is recommended by most dietitians
Caffeine	Events >30 min = yes; 30 sec–30 min = probably; repetitive sprints = no	Dependency, withdrawal, CNS stimulant effects, mild diuretic (effect *diminished* by exercise)	2–9 mg/kg (150–700mg) PO 1h prior to event	Mechanism of action is adenosine-receptor antagonism; enhanced fat metabolism, which spares glycogen stores, is possibly involved
Creatine	Repetitive maximum cycling bouts = yes; repetitive bouts weight lifting = probably; isometric strength, running, swimming, endurance activity = no	Weight gain due to water retention, ↑ intra-compartment pressure in lower extremities, renal and muscle dysfunction	20 g/day PO for 5 days; 2–5 g/day for maintenance	Ergogenic effect related to *increased phosphocreatine* stores; has not been shown to directly enhance protein/muscle synthesis; high individual *variability*
Ephedrine (*ephedra*, Ma Huang) and pseudoephedrine	Anaerobic activity = yes; endurance events = possibly	Hypertension, tachycardia, stroke, CNS over-stimulation	Ephedrine: 1 mg/kg 1h prior to event. Pseudoephedrine: 60–180mg 1h prior to event	Mechanism due to CNS arousal as opposed to muscle metabolism
Erythropoietin (recombinant)	Yes	Hypertension, seizures, *thromboembolic events*	50 IU/kg IV or SC, typically for 15–30 days	Improves oxygen carrying capacity of blood; extremely expensive and must be injected
β-Hydroxy-β-methylbutyrate (HMB)	Untrained individuals = possibly; trained individuals = no	Safe in short-term studies; few studies done	3 g/day PO	HMB is a metabolite of the essential amino acid leucine; promoted as an anti-catabolic
Human growth hormone (HGH)	Performance studies lacking; ↓ adipose tissue and ↑ *lean* body mass in a study in elderly men	Insulin resistance, glucose intolerance, oedema, ↓ endogenous HGH secretion	For medical conditions, 6–12 μg/kg SC daily. Anecdotal reports that abusers use much higher doses	Available only as a prescription SC injection. OTC products contain precursors or homeopathic amounts (600ng) of HGH given sublingually or nasally (unproven methods of delivery)
Proteins, amino acids	No	Excess protein stored as fat; theoretical concerns about renal load	Variable doses PO	A proper diet is all that is needed, even for strength athletes, to get the protein they need
Pyruvate	No	Unstudied	5 g/day PO	A carboxylic acid produced by the metabolism of glucose; no ergogenic effects even with high doses
Steroid precursors: androstenedione (AND); dehydroepiandrosterone (DHEA)	No	↓ high-density lipoprotein, ↑ estradiol/estrogen	AND: studies performed on 100–300 mg/day PO (product labels often instruct to take more); DHEA: 25 mg/day PO	Although testosterone/androgen precursors, do not promote protein/muscle synthesis. Can *create* positive drug tests for testosterone

IV = intravenously; **OTC** = over-the-counter; **PO** = orally; **SC** = subcutaneously; ↑ = increase; ↓ = decrease.

majority of anabolic-androgenic OTC products evaluated contained less product than the amount stated on the label.[1]

Despite what they may tell you ...

Pyruvate (table 1) is marketed as a performance-enhancing supplement for endurance

Table 2. Efficacy of β-hydroxy-β-methylbutyrate (HMB) in small placebo (PL)-controlled studies

Reference	HMB dosage (g/day)	Duration (wks)	Study population	Effect of HMB
Knitter et al.[10]	3	6	Untrained individuals	May reduce muscle damage; after a 20km run, lower increases in creatine phosphokinase and lactate dehydrogenase were shown with HMB than with PL
Gallagher et al.[11]	3 or 6	8	Untrained men undergoing resistance training (RT)	Results were mixed. One-repetition maximum strength did not improve in any group, and there were no between-group differences in body fat. Greater increases in peak isometric torque and fat-free mass were shown with 3 g/day than with either 6 g/day or PL
Panton et al.[12]	3	4	Untrained individuals undergoing RT	Increased upper body strength, but not lower body strength, compared with PL
Jowko et al.[13]	3	3	Untrained men undergoing RT	Improved results of 6 out of 7 strength tests compared with PL, but didn't significantly alter body fat or lean body mass
Slater et al.[14]	3	6	Trained men	No effect on strength or body composition

events, and also as a weight-loss and cholesterol-lowering agent.[1] It is suggested, but not proven, that pyruvate enhances glucose oxidation.[1]

... pyruvate is not ergogenic

In a placebo-controlled trial in active or trained individuals, pyruvate did not improve cycling performance times, blood pyruvate levels did not increase and there were no effects on carbohydrate or lipid metabolism.[8]

EPO can increase oxygen in blood ...

Erythropoietin (EPO), a hormone produced by the kidneys to regulate red blood cell production, is approved for medical use in chronic renal failure and certain types of anaemia.[1] Recombinant human EPO (r-HuEPO; table 1) is ergogenic due to its ability to improve the blood's oxygen carrying capacity,[9] and is often abused in the sporting world. In a 26-day study in nine athletes, r-HuEPO improved maximal oxygen consumption (VO_{2max}) by 9% and power output by 7% compared with baseline; maximum heart rate decreased by 5%.[9]

... but it could kill you

EPO is associated with serious thromboembolic events due to the increased packed red blood cell count and viscosity after administration.[1] The early deaths of several athletes have been attributed to abuse of EPO. The use of exogenous EPO is banned by the majority of major sporting associations. However, current tests to detect abuse have limited efficacy; further research into more sophisticated detection methods is required.[1]

Equivocal results with HMB ...

β-Hydroxy-β-methylbutyrate (HMB) [table1], a metabolite of leucine, is marketed as a supplement which increases lean body mass and strength by preventing muscle breakdown.[1] However, this promoted effect is yet to be proven; results have been variable in placebo-controlled studies of HMB (see table 2).[10-14]

... but it's safe so far

In one study, lipid profiles, hepatic enzyme levels and renal function were not adversely affected by HMB supplementation.[15] In studies of up to 8 weeks in length, no adverse effects were seen with HMB.[16]

HGH and IGF-1 unproven ergogenic effects

Human growth hormone (HGH) [table 1] is synthesised by the pituitary gland.[1] In a study in men aged ≥60 years, HGH increased lean body mass, decreased adipose tissue and slowed thinning of the skin;[17] however, as yet, there is no evidence to support the suggestion that HGH supplementation in athletes is ergogenic.[1] HGH is only available as an injection because the molecule it too big to be absorbed in the gastrointestinal tract.

Insulin-like growth factor-1 (IGF-1) is a hormone that is regulated, in part, by HGH.[1] Anabolic effects of IGF-1 have not been confirmed in humans. In one trial, individuals who doubled their baseline circulating IGF-1 levels experienced no increase in strength and no change in the rate of protein synthesis.[18]

OTC products not the real thing

Several OTC products that are claimed to contain HGH or IGF-1 actually contain precursors or alternative formulations that have not been proven to be performance enhancing.[1] The tolerability of these products has not been studied.[1]

Beware black market HGH

Prescription HGH (table 1) and IGF-1 are associated with serious adverse events.[1] Subcutaneous injections of IGF-1 are associated with temporomandibular joint discomfort, weight gain, dyspnoea and sinus tachycardia. Elevated IGF-1 levels may increase the risk of lung or colorectal cancer. HGH available on the black market is pituitary (not recombinant) derived, which raises concerns about the possibility of Creutzfeldt-Jakob disease or other contamination.

Essential dietary components …

Protein, branched chain amino acids and antioxidants (table 1) are key dietary components for athletes and non-athletes.[1] Protein and amino acid supplements are heavily marketed to athletes. Studies have shown that adding supplemental protein or branched chain amino acids (leucine, isoleucine and valine) to a normal diet offer no strength or muscle mass advantages to individuals.[1] In several studies, short-term supplementation with antioxidants did not improve VO_{2max} muscle energy metabolism, muscle fatigue, marathon running performance, muscles soreness or muscle damage.[1]

… but supplementation unnecessary

It is recommended that athletes increase their daily protein intake to 1.2–1.8 g/kg/day from the standard adult recommendation of 0.8 g/kg/day, and eat a diet rich in antioxidants.[1] In most cases, a well balanced diet is sufficient and supplementation is not necessary.[1]

Caffeine makes you faster …

Caffeine appears to have ergogenic potential, especially in aerobic activity (table 1).[1] In trained athletes, caffeine 2.1, 3.2 or 4.5 mg/kg all improved performance in a 1-hour cycling time trial.[19] The middle and highest dosages had similar efficacy. In another trial, caffeine 6 or 9 mg/kg enhanced the performance of well trained athletes who rowed 2000m.[20] Caffeine has also shown ergogenic potential in cycling and swimming sprints, but not in repetitive bouts of sprinting.[1]

… but don't overdo it

The IOC will disqualify athletes for having a urine caffeine concentration of 12 μg/mL: consumption of approximately 9 mg/kg will achieve this concentration.[1] In the above-mentioned rowing trial, urinary caffeine concentrations reached as high as 14 μg/mL.[20] However, in the cycling trial, urinary concentrations reached no higher than 2.5 μg/mL.[19] Caffeine is generally taken in the form of tablets; the amount of caffeine in coffee is approximately 100–250 mg/250mL.[1]

Creatine results variable

Supplementation of creatine (table 1) supposedly increases creatine and phosphocreatine concentrations in skeletal muscle, allowing greater adenosine triphosphate availability during intense anaerobic exercise.[1] The ergogenic effect of creatine has been frequently studied, with variable results.[1] Creatine was ergogenic in individuals undergoing repetitive bouts of high-intensity cycling and repetitive short running sprints.[1] However, creatine supplementation did not enhance isometric strength, overhead motion strength or performance in running or swimming sprints.[1]

The extra weight may slow you down

Two well documented adverse effects of creatine are weight gain and increased muscle compartment pressure.[1] Serum creatine levels and glomerular filtration rate may also be affected.[1] The rapid weight gain caused by creatine may be detrimental to running or swimming performance.

Ephedrine can be ergogenic …

Ephedrine and pseudoephedrine (table 1) are sympathomimetic amines with stimulant properties. They are used by athletes to increase energy and delay fatigue and by wrestlers to reduce body mass prior to competition.[1] Ephedrine 0.8 mg/kg improved anaerobic performance in prolonged exercise.[21] Pseudoephedrine 120 or 240 mg/day has not shown ergogenic effects in aerobic or endurance activity;[1] however, pseudoephedrine 180mg was ergogenic in anaerobic burst-type activity.[22]

… but has cardiovascular effects

There have been numerous reports of adverse cardiovascular and CNS events associated with pseudoephedrine and ephedrine.[1] There have also been a number of deaths and permanent disabilities associated with supplements containing ephedra alkaloids. The use of pseudoephedrine or ephedrine is banned by most sporting associations.[1]

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PRODUCT INTRODUCTIONS

New in the Marketplace

The following table contains a summary of product launches and approvals identified in international markets in recent weeks by the drug therapy reporting service *Inpharma Weekly*.[1]

Generic name	Trade name (Company)	Indication/Category	Country
Atazanavir (A)	Reyataz (Bristol-Myers Squibb)	Treatment of HIV infection	EU
Atazanavir (L)	Reyataz (Bristol-Myers Squibb)	Treatment of HIV infection	UK
Bevacizumab (L)	Avastin (Genentech)	Treatment of colorectal cancer	US
Ciclesonide (A)	Alvesco (Altana Pharma)	Treatment of asthma	Australia
Cinacalcet (A)	Sensipar (Amgen)	Treatment of secondary hyperparathyroidism in chronic kidney disease and treatment of hypercalcaemia	US
Efalizumab (A)	Raptiva (Serono)	Treatment of moderate-to-severe psoriasis in adults	Switzerland
Everolimus (L)	Certican (Novartis)	Immunosuppressant	Germany
Ezetimibe/simvastatin (A)	N/A (Schering-Plough, Merck & Co)	Treatment of hypercholesterolaemia	Mexico
Fulvestrant (L)	Faslodex (AstraZeneca)	Treatment of breast cancer	Germany, Sweden
Gefitinib (A)	Iressa (AstraZeneca)	Treatment of advanced non-small-cell lung cancer	Switzerland
Tandospirone (L)	Sediel (Sumitomo Pharmaceuticals)	Treatment of anxiety disorders	China

A = approved; L = launched; N/A = not available

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[1] *Inpharma Weekly* provides rapid alerts to news on drugs and drug therapy. Summarising information selected from over 1600 biomedical publications, this newsletter is produced by Adis International Limited and is available in a variety of formats. Please contact your nearest Adis office for subscription details.

Introduction:

Competition and the drive for excellence motivated the application of biochemical physiology knowledge to athletic training regimens. One of the first such applications is that of creatine monohydrate (CrM) for world-class athletes, the use of which dates to the 1970's, when state-sponsored Olympic Games athletes from the Soviet Union used creatine as an ergogenic aid.[1] The prominent Ukrainian biochemist Olexander Pallandin, who trained under Ivan Pavlov, showed in the 1930's that levels of creatine and phosphocreatine change with contraction strength and exertion and are increased by training. He also showed levels are higher in fast-twitch, white fibers than slow-twitch, red fibers. Based upon this pioneering work, the research and subsequent use of creatine as an ergogenic dietary supplement for elite athletes was sponsored by the Central Institute of Physical Culture in Moscow.

The history of the use of CrM supplementation illustrates the culture of competitive athletics, wherein the pursuit of improved performance often overrides caution about possible health risks. Even amidst isolated case reports of severe toxicity and the absence of comprehensive long term studies of safety, creatine supplementation remains extremely common: Metzl et al. showed that 28% of collegiate athletes and many high school athletes, particularly 11th and 12th grade males, take creatinine.[2]

Biochemical Physiology:

The use of creatine monohydrate (CrM) as an athletic supplement is based on the physiological presence of creatine (Cr) and phosphoryl creatine (PCr) in skeletal muscle, where PCr acts as a minor store of high energy compounds, which may rapidly but only transiently be recruited to replenish the depleted energy stores in vigorously contracting muscles. The CrM supplement theory postulates that, by increasing the concentration of Cr in skeletal muscle, CrM will allow muscle to achieve a higher anaerobic threshold and to undergo higher intensity training.

Creatine is a non-essential amino acid that is formed in the liver, pancreas and kidney and also consumed in the diet from the ingestion of animal products. Cr has the ability to bind a high energy phosphate, forming PCr. During high intensity exercise, muscle drains its adenosine triphosphate (ATP) stores. PCr donates its high energy phosphate to adenosine diphosphate (ADP), leading to the re-synthesis of adenosine triphosphate (ATP). Thus, PCr acts as a short-term energy buffer during periods of rapid ATP turnover. The system is high power (large amounts of ATP may be produced) but low capacity (storage amounts are normally drained in less than 20 seconds), thus fitting a role in anaerobic activity.[9]

Skeletal muscle is the body's primary repository for Cr, and thus is the target for CrM supplementation. Muscle Cr stores break down at a relatively constant rate of approximately 2 grams/day into creatinine. Cr is normally filtered at a consistent rate by the kidney, thus making Cr a useful measure of kidney function.

Purported Benefits:

CrM is generally considered an effective enhancer for high intensity, short duration activity but not for longer duration, endurance / aerobic activity. Over 500 research studies have examined the effect of CrM on athletic performance. Most studies on the potential value of CrM as an ergogenic augmenter report statistically significant gains in creatine takers.[16] A number of recent randomized controlled trials of selected populations of athletes confirms this finding. Mero et al. show that interval swimming performance is improved with CrM.[18] Ostojic demonstrated that soccer-specific skill performance is enhanced with CrM.[19] Biwer et al. showed that submaximal running interspersed within high intensity intervals is not improved with CrM.[23]

Given the normal physiological role of PCr in skeletal muscle, CrM is used therapeutically for a wide variety of diseases. Recent studies have confirmed the efficacy of CrM for selected diseases. Tarnopolsky et al. report that the use of CrM enhances muscle strength and fat free mass in children with Duchenne muscular dystrophy.[17] Lebacq et al. corroborated this by showing improved strength and bone mineral density and decreased fatigue in patients with Duchenne and Becker dystrophy.[22] Korenke et al. report that CrM led to long term improvement for a girl with ataxia and weakness due to a long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency.[21] However, Bohnhorst et al. report that the use of CrM in infants with apnea of prematurity is not clinically helpful.[20]

Side Effects:

The question of whether creatine poses short or long term health risks remains unresolved. In the absence of large scale, long term randomized study, the safety of the routine use of creatine among young athletes cannot be definitively confirmed. Two key risks categories exist today: 1) the potential for direct toxic injury and 2) the lack of quality control.

Isolated cases have implicated creatine supplementation in direct renal toxicity.[3,4,5] However, no large scale studies have shown a pattern of renal or other toxicity in healthy humans.[6] Researchers agree that longer term studies are necessary before the possibility of a pattern of toxicity can eliminated. However, most striking in the literature is a general discord among researchers as to whether current studies suggest that creatine represents a serious risk to tissue injury. Direct and indirect evidence exists to support both the argument for its potential toxicity as well as that for its innocuousness. In Nutrition Bytes 2002, Der Hovanessian commented that studies failed to show evidence for toxicity due to short and medium term use of CrM, but he reserved caution for the potential for toxicity due to long term use.[10] This caution stemmed from the lack of reliable studies, with only the 5 year study by Poortmans et al., qualifying as long term.[11] While this study reported no major toxicity, among other problems, it only had 9 study subjects. The fact that the Poortmans et al. might be considered a substantial study highlights the paucity of reliable evidence on creatine use. The major limitations of research to date are small sample size and short length of studies. If toxicity is rare, large scale studies are required to capture the effect. Additionally, if the effects are insidious and slow in onset, only longer term studies will sufficiently recognize the deleterious effects. It cannot be argued that studies to date offer strong evidence-based artillery either to the industry proponent nor the frank critic of creatine use.

In terms of indirect evidence, the results of animal studies are mixed. Taes et al. report that 28 day treatment of both healthy and partially nephrectomized rats with creatine supplementation did not impair renal function.[7] A study by Edmunds et al. challenges this conclusion. It examined creatine supplementation in rats with cystic kidney diseases and showed that creatine may exacerbate the progression of kidney disease.[14] Meanwhile, Tarnopolsky et al. report mixed results after 159 day treatment of mice and 365 day treatment of rats. While histological examination of rat livers revealed no abnormalities, that of the mice livers showed areas of hepatitis.[8] Also, studies of creatine metabolism suggest that its breakdown may lead to mutagenic metabolites.[9] The implication of an increased carcinogenic load due to creatine and its metabolites cannot be overlooked, notwithstanding the lack of specific studies confirming the causal or contributory relation between creatine and certain cancers.

Results from direct studies are equally mixed and not of sufficient quality to address the concern of potential long term toxicity. Two recent studies of moderate length argue for the intermediate term lack of severe toxicity of creatine. Kreider et al examined a 69 item panel of serum, whole blood and urinary markers of clinical health, including metabolic markers, muscle and liver enzymes, electrolytes, lipid profiles, hematological markers and lymphocytes, in college athletes in a 21 month non-blinded trial.[12] This trial failed to show significant differences in clinical markers between creatine takers and controls. In a companion study, Greenwood et al. compared the incidence of cramping, dehydration, muscle tightness, and injuries in creatine takers and controls among college athletes in a 3 year non-blinded trial.[13] This study found fewer or similar rates of the above problems among creatine takers when compared to controls.

The second major risk category is lack of quality control. Creatine supplementation remains relatively unregulated. According to the Dietary Supplement Health and Education Act of 1994, much less stringent oversight exists for nutritional supplements, such as creatine, than for pharmaceuticals, allowing food supplements producers to make structure and function claims without FDA approval or comprehensive scientific backing. Equally alarming is the lack of assurance for integrity of concentration and purity, leaving open the possibilities of toxicity due to overdosing and contamination.[15]

Conclusion:

The use of creatine supplementation continues to be a hotly debated and relevant topic for health care professionals, amid its widespread use among young people and the mass of research with inconclusive or questionable outcomes. While the argument that creatine is an effective anaerobic ergogenic aid has a wealth of evidence, the case for the safety of its long term use does not. This fact is worrisome given the cavalier manner in which many use the supplement, as considerations of genetic predispositions to kidney disease, underlying occult or known disease states, or even metabolically competing medications are often excluded from the decision of whether, how much and for how long to use the creatine supplement. While evidence to date does not suggest a widespread pattern of severe side effects from its use, the research is silent regarding risks in the context of disease, such as impaired glomerular filtration. In an era where the burden of proof lies with the health care professional, who operates under the principle of evidence based medicine, physicians should work to communicate this lack of clarity, to urge caution and to demand more comprehensive research.

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Long-term dietary L-arginine supplementation attenuates proteinuria and focal glomerulosclerosis in experimental chronic renal transplant failure

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Abstract

Glomerular endothelial nitric oxide synthase expression is decreased in humans during acute rejection and chronic renal transplant failure (CRTF). This may contribute to vascular damage through changes in the renal hemodynamics and enhanced endothelial adhesion of leukocytes and platelets. Dietary supplementation of L-arginine may increase endothelial NO production, thereby protecting the vascular wall and improving renal hemodynamics. We tested the hypothesis that long-term L-arginine supplementation attenuates the development of CRTF in an experimental model for renal transplantation. In the Fisher 344 to Lewis rat model for renal transplantation, renal function and histology of untreated rats was compared with rats receiving L-arginine in the drinking water (10 g/L), starting 2 days before transplantation. Every 4 weeks systolic blood pressure was measured and serum and urine were collected for measurement of nitrite and nitrate (NO_x), creatinine, and proteinuria. At 34 weeks the histological renal damage was assessed by scoring focal glomerulosclerosis and measurement of alpha-smooth muscle actin (α-SMA) expression. Urinary NO_x was significantly increased in treated animals. Proteinuria was significantly lower in L-arginine-treated animals from week 24 onward ($p < 0.05$). Plasma creatinine and creatinine clearance did not differ between the groups. The focal and segmental glomerulosclerosis (FGS) score (max 400) at week 34 was also significantly lower in treated rats arbitrary U (20 ± 21 vs 61 ± 67 arbitrary U; $p < 0.05$). The expression of α-SMA was lower in L-arginine-treated rats than in untreated rats ($1.93 \pm 0.8\%$ area surface vs $3.64 \pm 2.5\%$ area surface). In conclusion, in this experimental model for CRTF, L-arginine administration significantly reduced FGS and proteinuria, without affecting renal function. Our data suggest that dietary L-arginine supplementation attenuates progression of CRTF and may therefore be an additional therapeutic option in human renal allograft recipients.

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The initiation and progression of chronic renal graft dysfunction is mainly determined by the extent of vascular injury and proliferation. Important for optimal functioning and protection of the endothelium is the production and availability of nitric oxide (NO). Endothelial-derived NO regulates vascular tone [1] but also inhibits leukocyte adhesion to the endothelium [2], prevents platelet aggregation [3], and inhibits proliferation of vascular smooth muscle cells [4]. NO is formed by hydroxylation of L-arginine (L-Arg) to N^G-hydroxy-L-arginine and subsequent structural oxidation to L-citrulline and NO [5], a process which is catalyzed by nitric oxide synthases (NOS) [6].

In several renal diseases in humans, such as acute renal failure [7], acute rejection [8], and chronic renal transplant failure (CRTF) [9], endothelial NO synthase (eNOS) expression and NO availability is reduced. In several experimental models for acute or chronic renal failure, renal function improved and histological damage decreased upon L-Arg supplementation [10,11]. In an experimental model for renal transplant failure, L-Arg improved renal function and pathology in an early stage [12]. In humans, data on L-Arg supplementation is rather inconsistent. While some studies report no effect

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of L-Arg on renal function [13,14], a recent study by Schramm et al. [10] did show beneficial effects of L-Arg supplementation on glomerular filtration rate and renal plasma flow. In conclusion, short-term L-Arg supplementation is renoprotective in some diseases and in renal transplantation. However, no study has investigated the effect of long-term dietary L-Arg supplementation on the development of CRTF.

The aim of our study was to increase L-Arg availability and consequently increase intrarenal NO levels systemically in an experimental model for CRTF by dietary supplementation of L-arginine. Thereby we expect to improve graft structure and function and to attenuate the development of CRTF.

Methods and materials

Experimental techniques

Inbred male rats, weighing 248 ± 6g were used (Harlan, United Kingdom). All animals were housed in a temperature-controlled environment with a 12-h light–dark cycle. The rats had free access to drinking water and standard rat chow. Renal transplantation was performed according to standard procedures [15]. Lewis rats (Lewis SsnHsD) served as recipients, Fisher 344 rats served as donors. The rats were anesthetized with isoflurane; left donor kidneys were flushed with saline, preserved in saline on ice for about 20 min, and transplanted orthotopically. The left renal vessels and ureter were anastomosed end-to-end using 10-0 prolene sutures (Johnson & Johnson Int., Brussels, Belgium). Vascular clamps were released immediately after the vascular anastomosis was completed, with a warm ischemic time of 20 ± 5 min. A short course of cyclosporine A (CsA; 1.5 mg/kg/day, Sandimmune; Sandoz Pharma AG, Bazel, Switzerland) was administered subcutaneously for a period of 10 days to all rats to reverse early acute rejection episodes. After 10 days the native right kidney was removed and the transplanted kidney was inspected for viability and hydronephrosis.

Experimental groups

The experimental groups consisted of 10 untreated allografted rats (F-L) and 10 allografted rats receiving drinking water supplemented with 1% L-arginine (F-L + L-Arg; 10 g/L) (both Sigma–Aldrich, Zwijndrecht, Netherlands). At day 10 following transplantation 4 rats were excluded from the study due to development of hydronephrosis (1 F-L and 3 F-L + L-Arg). L-Arg supplementation started 2 days before transplantation and was given to both the donor and the recipient.

Clinicopathological parameters

The transplanted rats were followed for 34 weeks. Body weight was measured every 4 weeks. Urine was collected every 4 weeks by placing the rats for 24 h in metabolic cages without access to food. Blood samples were collected every 4 weeks from the tail vein. Systolic blood pressure (SBP) was measured every 4 weeks using the tail cuff method while rats were anaesthetized with isoflurane. During follow-up water intake was measured every 2 months by measuring the individual total water intake over several days. At the end of the observation period, the rats were anaesthetized with isoflurane, SBP was measured, a 2-ml blood sample was taken by canulation of the aorta, and the kidney was perfused in situ with saline and removed.

Tissue processing and staining procedures

Immediately after perfusion, a coronal tissue slice through the midportion of the kidney was snap-frozen in liquid isopentane and stored at −80 °C for immunohistochemistry. Another coronal tissue slice was fixed in 4% paraformaldehyde and processed for paraffin embedding. Paraffin sections were stained with periodic acid-Schiff and Verhoeff to evaluate glomerular, interstitial, and vascular damage.

Immunohistochemistry

Paraffin sections (4 μm) were dewaxed and subjected to heat-induced antigen retrieval by overnight incubation in 0.1 M Tris/HCl buffer at 80 °C. Cryostat sections were cut at 4 μm and fixed in acetone for 10 min. Endogenous peroxidase was blocked with 0.075% H_2O_2 in phosphate-buffered saline (PBS) for 30 min. Alfa-smooth muscle actin (α-SMA) and eNOS were detected using a mouse monoclonal α-SMA antibody (clone 1A4; Sigma Chemical, St. Louis, MO) and a mouse monoclonal anti-eNOS antibody (Transduction Laboratories, Lexington, KY; Cat. N30020), respectively. Nitrotyrosin was detected using a polyclonal anti-nitrotyrosin antibody (Upstate Biotechnology, Lake Placid, NY; Cat. N06-284). First antibodies were incubated for 60 min. Binding of the antibody was detected using sequential incubations with peroxidase (PO)-labeled rabbit anti-mouse and PO-labeled goat anti-rabbit antibody (both from Dakopatts, DAKO, Glostrup, Denmark) for 30 min. Antibody dilutions were made in PBS supplemented with 1% bovine serum albumin and 1% normal rat serum. Peroxidase activity was developed by using 3,3′-diaminobenzidine tetrachloride for 10 min for detection of α-SMA or by using a freshly prepared solution of 3-amino-9-ethylcarbimazole and H_2O_2 for eNOS and nitrotyrosin detection. Sections were counterstained with hematoxylin.

The expression of glomerular α-SMA and eNOS was measured using computerized morphometry. All viable, nonsclerosed glomeruli were used for glomerular measurements of α-SMA and eNOS staining. The image of a given glomerulus present on the screen was traced with a cursor along Bowman's capsule over the surface of a graphic tablet connected to the computer. For interstitial measurement of α-SMA, 50 fields were scored; vessels and glomeruli were excluded from measurement. The renal sections were evaluated moving from cortex to medulla and vice versa at a magnification of 20×. The total staining was divided by the total area surface and expressed as a percentage. The average score was calculated per cortical section.

Measurements in blood and urine

Urinary protein (UP) was measured using the pyrogallol red molybdate method. Combined total nitrite and nitrate concentrations (NO_x) in urine were measured using the Griess reaction after reduction of nitrate to nitrite [16,17]. Urinary NO_x concentrations were expressed in relation to urinary creatinine concentrations. Plasma and urine creatinine levels were determined colorimetrically (Sigma Chemical).

Analysis of structural changes

The severity of focal and segmental glomerulosclerosis (FGS) was scored semi quantitatively on a scale of 0 to 400. FGS was scored positive if mesangial matrix expansion and adhesion formation were present in the same quadrant. If 25% of the glomerulus was affected a score of 1+ was adjudged; 50% was scored as 2+, 75% as 3+, and 100% as 4+. The ultimate score was obtained by multiplying the degree of injury by the percentage of glomeruli with the same degree of injury and addition of these scores. A total of 45–50 glomeruli per animal were scored, moving from cortex to medulla and vice versa.

Statistics

Data are expressed as mean ± SD. Comparisons between two groups were made by Student *t* test. Levels of proteinuria and blood pressure in the treated and untreated animals were compared with repeated-measurements analysis of variance. Differences were considered statistically significant at a two-sided $p < 0.05$.

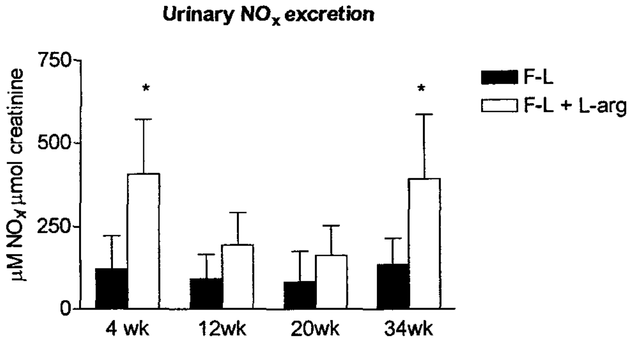

Fig. 1. Urinary NO_x excretion during the 34-week follow-up; $^*p < 0.05$.

Results

L-arginine uptake

Supplementation of the drinking water with 1% L-Arg resulted in an average intake of 1 mg L-Arg/day/g. There was no difference in drinking behaviour between the two groups. The L-Arg supplementation resulted in a significant increase in the urinary concentration of nitrate and nitrite (NO_x). At all time points measured, during the 34-week follow-up urinary NO_x excretion was higher. At 4 and 34 weeks after transplantation, urinary NO_x excretion was even significantly higher in rats receiving L-Arg than in untreated rats ($p < 0.05$; Fig. 1).

Clinical parameters

Mean values of the clinical parameters are given in Table 1. Body weight was not different between the two groups. Fig. 2 shows the time course of up excretion in the two groups. In untreated allografts proteinuria developed around week 24. Rats that received L-Arg had significantly lower levels of urinary protein excretion from week 24 onward. Systolic blood pressure was not significantly different between the groups during the follow-up (Fig. 3). Plasma creatinine and creatinine clearance also did not differ between the groups (Table 1, Fig. 4).

Histological parameters

Focal glomerulosclerosis was significantly reduced in the L-Arg group (20 ± 21.3 arbitrary U (AU)) compared

Table 1
Clinical parameters at week 34

Group (*n*)	Body weight (mg/24 h)	SBP (mm Hg)	Plasma creatinine (µmol/L)	Creatinine clearance (µl/min)
Allograft (9)	381 ± 20	128 ± 11	59.9 ± 21.3	1.84 ± 0.92
Allograft + L-arginine (7)	402 ± 18	122 ± 14	54.2 ± 6.6	1.53 ± 0.32

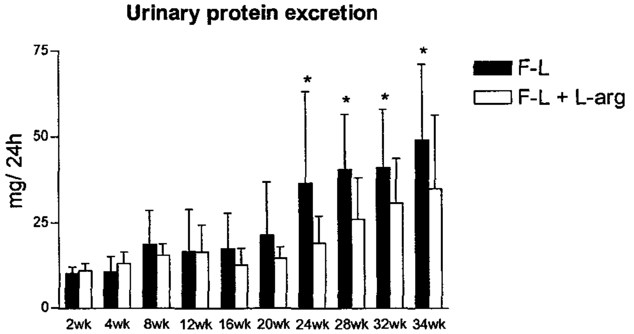

Fig. 2. Time course of urinary protein excretion in F-L rats vs F-L rats receiving L-arginine. Values are presented as means ± SD; *$p < 0.05$.

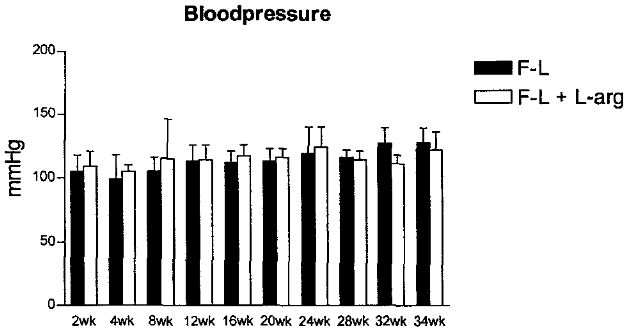

Fig. 3. Time course of systolic blood pressure in F-L rats vs F-L rats receiving L-arginine. Values are presented as means ± SD.

to the untreated group (61.3 ± 67 AU; see Fig. 5). The expression of interstitial α-SMA was lower in the L-Arg-treated rats than in the untreated rats (1.93 ± 0.8% area surface vs 3.64 ± 2.5% area surface; $p < 0.07$). Glomerular α-SMA was not different between the two groups. Using the Verhoeff staining, we examined the intrarenal arteries for vascular abnormalities. In both the control rats and the L-Arg-treated rats no vascular abnormalities could be found. Nitrotyrosin expression was absent in both groups. The expression of glomerular eNOS was identical in both groups (data not shown).

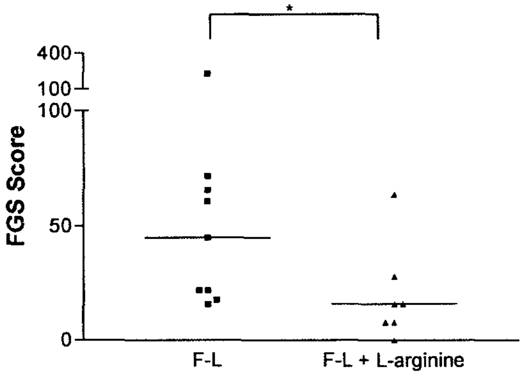

Fig. 5. FGS injury scores in F-L rats vs F-L rats receiving L-Arg; *$p < 0.05$.

Discussion

This study demonstrates a beneficial effect of nitric oxide modulation by long-term supplementation of L-Arg in an experimental model for CRTF. We show that L-Arg supplementation leads to an increase in NO production as measured by an increase in urinary NO_x excretion, accompanied by a significant reduction in proteinuria and a significant reduction in glomerulosclerosis.

Endothelial injury can lead to endothelial dysfunction and consequently to an impaired NO availability. This may result in vascular damage caused by an increase in leukocytes and platelet adhesion to the vascular wall, a disturbance in renal hemodynamics, and proliferation of the VSMC. Endothelial dysfunction seen in renal transplant patients may be the result of events such as ischemia/reperfusion (I/R) injury, immunological damage, and cyclosporine toxicity [18,19]. Furthermore, classical risk factors such as hypertension and hypercholesteremia may also contribute to endothelial dysfunction. By increasing L-Arg availability, endothelial NO production increases, which helps to protect the endothelium and thereby restore endothelial function.

Our study is the first to demonstrate a beneficial long-term effect of L-Arg in an experimental model for renal

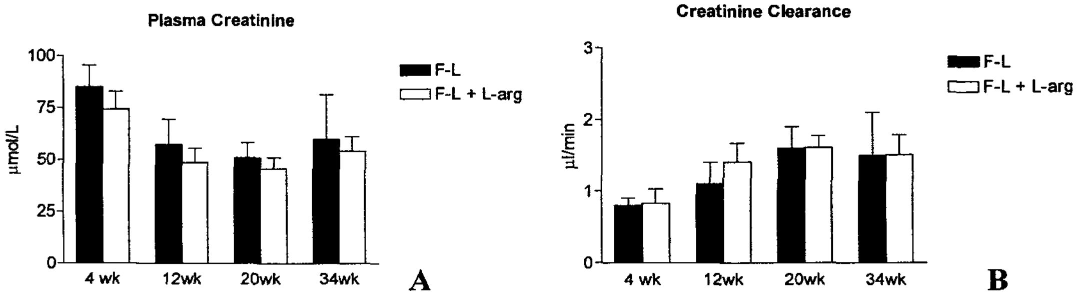

Fig. 4. (A) Plasma creatinine levels and (B) creatinine clearance levels during the 34-week follow up. Values are presented as means ± SD.

transplantation. So far, studies have looked at the effect of L-Arg in the acute phase of renal transplantation and not at the long-term effects [12]. Long-term studies performed in the renal ablation model, which shows strong histological similarities with CRTF, as it is also characterized by progressive glomerulosclerosis, interstitial fibrosis, and proteinuria, revealed improved renal histology and function after long-term L-Arg supplementation [20–22].

The beneficial effects of L-Arg are not restricted to renal transplantation alone. L-Arg also reduces liver and biliary tract damage after liver transplantation [23,24] and it improves endothelial dysfunction in cardiac transplant patients [25]. In addition, L-Arg prevents renal damage in other experimental models such as acute renal failure [11] and cyclosporine toxicity [18,19].

Our findings are also in line with a recent study by Vos et al. [12] who found a beneficial effect of L-Arg supplementation on glomerular filtration rate, renal plasma flow, and renal pathology 7 days posttransplantation. In this study only a low dose of CsA was given, yielding a mild acute rejection. The effects of L-Arg are therefore more related to protection of graft against immunological damage in the early phase after transplantation. In our model, proteinuria levels do not differ until week 20, which suggests that renal function is unchanged up to 20 weeks. Therefore the effect that we demonstrated is unlikely to result from a protective effect of L-Arg during the early stage alone. It is more likely that L-Arg supplementation is protective against immunological and I/R damage during the first weeks posttransplantation but remains protective weeks or months after transplantation by a constant higher availability of endothelial NO.

Some caution should be taken in L-Arg supplementation, since L-Arg can also enhance renal damage in iNOS-dependent renal injury as seen in inflammatory diseases such as glomerulonephritis [26,27]. Interaction between O_2^- and NO may lead to the formation of peroxynitrite ($ONOO^-$) [28–30], a cytotoxic molecule capable of initiating lipid peroxidation, and nitration of tyrosine residues (nitro tyrosine), resulting in loss of protein structure and function [31]. In our model CsA inhibits acute rejection and thus severe inflammation; therefore iNOS-induced NO production and production of O_2^- by inflammatory cells is minimal. Also, we could not find an increase in nitrotyrosine formation, which suggests that in our model L-Arg did not enhance renal damage, caused by high NO production by iNOS.

In conclusion, in the experimental renal transplant model, L-arginine supplementation attenuates progression of CRTF and may therefore be an additional therapeutic option in human renal allograft recipients.

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ORIGINAL ARTICLE

Deleteriuos effects of immobilization upon rat skeletal muscle: role of creatine supplementation

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KEYWORDS
Creatine;
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Skeletal muscle

Summary *Aim:* The aim of the study was to investigate the impact of creatine feeding ($5\,g\,kg^{-1}$ body weight day^{-1}) upon the deleterious adaptations in skeletal muscle induced by immobilization.

Methods: Male Wistar rats were submitted to hind limb immobilization together with three dietary manipulations: control, supplemented with creatine for 7 days (along with immobilization) and supplemented with creatine for 14 days (7 days before immobilization and together with immobilization). Muscle weight (wet/dry) was determined in the soleus (SOL) and gastrocnemius (GAS). The analysis of lean mass was performed by DEXA and myosin heavy chain (MHC) distribution by SDS-PAGE.

Results: After 14 days of creatine loading, immobilized SOL and GAS total creatine content were increased by 25% and 18%, respectively. Regardless of dietary manipulation, the immobilization protocol induced a decrease in the weight of SOL and GAS ($P<0.001$). However, creatine feeding for 14 days minimized mass loss in the SOL and GAS ($P<0.05$). Our findings also indicate that creatine supplementation maximizes the expected slow-to-fast MHC shift driven by immobilization ($P<0.05$).

Conclusions: Previous creatine supplementation attenuates muscle wasting induced by immobilization. This effect is associated with the increment of intramuscular creatine content.

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Introduction

The deleterious effects of hypokinesia upon skeletal muscle have been intensely investigated for over 40 years. Generally, hypoactivity induces muscle atrophy, slow-to-fast twitch shift and a decrease in force generation capacity.[1–6] These adaptations have been observed in various conditions, such as immobilization,[7] hind limb suspension[3] and microgravity,[8] although it is clear that other specific modifications in muscle function occur in these models.

Creatine is an organic compound that is directly involved in the muscle energy buffering system.[9] When phosphorylated by creatine kinase, the

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resulting phosphocreatine can donate inorganic phosphate and energy in order to rephosphorylate ADP, thus providing the proper energetic environment to the skeletal muscle contractile apparatus.[9] Creatine loading has also been widely studied due to its potential ergogenic effect in sports performance, for example in increased power output and muscle mass gain.[9] In addition, recent studies have shown that creatine can enhance muscle functional capacity in patients with neuromuscular diseases or muscular dystrophies.[10,11]

Experimental evidence indicates that creatine might modulate skeletal muscle tropism in specific conditions. For example, recent studies have shown that creatine feeding induces satellite cell proliferation[12] and expression of specific muscle transcriptional factors.[13] Vierck et al.[14] were able to show a direct effect of creatine on satellite cells in culture; it was observed that the cells differentiated earlier and to a greater level. In line with a stimulatory effect of creatine on satellite cells, Dangott et al.[12] demonstrated higher satellite cell mitotic activity in creatine-fed rats submitted to plantar skeletal muscle hypertrophy in the ablation model.

Another study conducted by Hespel et al.[13] investigated the effects of creatine supplementation during immobilization and rehabilitation on the expression of muscle myogenic factors in humans. This study revealed an over expression of muscle regulatory factor–4 (MRF-4) in the creatine-fed group during recovery. These findings suggest that creatine can also modulate skeletal muscle plasticity by molecular mechanisms. Still the mechanisms underlying the beneficial effects of creatine loading on skeletal muscle remain largely unknown.

Given that creatine is involved in many processes that regulate skeletal muscle tropism, we aimed to investigate whether creatine loading is able to attenuate the deleterious effects of disuse. The identification and characterization of strategies prone to minimize the effects of skeletal muscle disuse, will certainly contribute to a better outcome of a large number of individuals subjected to hypokinesia.

Material and methods

Animals

Male adult Wistar rats, weighing 200–220 g, were obtained from the animal breeding facility of the Institute of Biomedical Sciences, University of Sao Paulo. The animals were maintained on a constant light-to-dark cycle (12 h:12 h) in metabolic cages, and received water and food ad libitum. The animals were anesthetized by intra-peritoneal injection of a mixture of ketamine ($30\,\mathrm{g\,kg^{-1}}$ body weight) and xilazine ($10\,\mathrm{g\,kg^{-1}}$ body weight) before the immobilization procedure. The following groups were studied: control supplemented with saline (C; $n=6$), supplemented with creatine during 7 days of immobilization (Cre7; $n=6$) and pre-supplemented with creatine for 7 days before immobilization and also subsequently for 7 days after the onset of immobilization (Cre14; $n=6$). In order to ensure that the contralateral leg was an adequate control reference, a fourth group of animals, non-immobilized age-matched controls (NI; $n=12$) was studied. The experimental protocol (# 097/2002) was approved by the Institute of Biomedical Sciences Ethical Committee for Animal Research, University of Sao Paulo.

Supplementation protocol

All groups were fed with standard rat chow. A high dose of creatine monohydrate was administrated daily via *gavage* ($5\,\mathrm{g\,kg^{-1}}$ body weight). In order to avoid gastrointestinal disturbance the daily dose was divided into three rounds. The control group (C) received the same volume of 0.9% saline.

Hind limb immobilization procedure

A mono-lateral hind limb immobilization was performed in all rats, with the exception of the NI group. The left limb of each animal was immobilized (I) with a cast on the total plantar extension.[15] Gastrocnemius (GAS) and soleus (SOL) muscles remained in shortened positions for 7 days. As previously described,[16] the contra-lateral leg that remained loose was utilized as a control for the immobilized leg of each animal. The legs were subdivided into: contra-lateral leg of control group (C-CL), contra-lateral leg of creatine-supplemented group (Cre7-CL), contra-lateral leg of creatine pre-supplemented group (Cre14-CL), immobilized leg of control group (C-I), immobilized leg of creatine-supplemented group (Cre7-I) and immobilized leg of creatine pre-supplemented group (Cre14-I). A fine-meshed steel net involved the cast to avoid chewing.[16] Great care was taken to make sure that the cast did not cause ischemia.

Measurement of total creatine content

The SOL and GAS total creatine content were determined by reversed-phase high-performance liquid chromatography (HPLC) technique. Frozen muscles were powdered in a stainless steel percussion mortar cooled in liquid nitrogen. The powder was homogenized in 0.4 N perchloric acid at 0°C and aliquots of the homogenate were removed for protein determination. Samples were centrifuged at 3000 *g* for 1 min and the supernatant buffered to pH 7.0 with 0.2 mol l^{-1} Tris and KOH, 40% weight per volume, in 60:40 mix. Further centrifugation at room temperature and 3000 *g* for 1 min was realized to remove the precipitate before 50 μl of the final supernatant was analyzed.[17]

The HPLC system consisted of auto-injector (SIL-10AD; Shimadzu, Tokyo-Japan), a liquid chromatograph (LC-10AD; Shimadzu, Tokyo-Japan) and UV–visible spectrophotometric detector (SPD-10A; Shimadzu, Tokyo-Japan). The column utilized was an Absorbosphere HSC18 (250 mm × 4.6 mm, 7 μm pore size; Alltech, Deerfield, USA). The column was perfused at 0.9 ml min^{-1} with a mixture of Mg^{2+}–Tris buffer containing $MgSO_4$ (4 mmol l^{-1}), Tris (4 mmol l^{-1}), KH_2PO_4 (22 mmol l^{-1}) and NaN_3 (0.2 mmol l^{-1}) and a solvent consisting of methyl alcohol: water mix, in a 60:40 volume per volume ratio at pH 6.05.[17] To improve retention times for PCr and Cr, samples were isocratically using a phosphate buffer (90 mmol l^{-1} KH_2PO_4, 10 mmol l^{-1} K_2HPO_4 (pH 4.75) containing an ion-pairing agent (5.9 mmol l^{-1} tetrabutylammonium hydrogen sulfate) that improves the retention of PCr.[18] In order to minimize mislead results, since high phosphate breakdown is extremely rapid and would not represent the animal living content, according to Robinson and Loiselle,[17] we report only the sum of Cr and PCr (Total creatine content).

Lean mass determination

Lean mass (LM), which is mainly composed of muscle mass, was evaluated by dual energy X-ray absorptiometry as described before by Brommage[19] using the pDEXA Sabre Bone Densitometer and Software (Norland Medical Systems©), both especially designed for small animals. The animals were scanned under anesthesia just before immobilization (basal scan—day 0) and after 7 days of immobilization (final scan—day 7). The lean mass changes were expressed in grams ($\Delta LM = LM$ day 7$-$LM day 0).

Tissue samples

The muscles (GAS and SOL) were removed and weighed for relative mass determination. Immediately afterwards the muscles were quickly frozen, and stored at −70°C for posterior determinations of MHC isoform distribution. To determine dry weight, a small aliquot of each muscle was weighed and then dried for 48 h at 60°C. The weight of adrenal glands was determined to estimate the level of stress in all experimental groups (C, Cre7 and Cre14) in relation to rats not submitted to immobilization (NI).

Myosin heavy chain analysis

The muscles were frozen in liquid nitrogen and stored at −70°C. Muscles samples preparation to electrophoretic separation were conducted as described before by Talmagde and Roy.[20] Proteins were separated by SDS-PAGE gels containing 30% vol/vol glycerol, 8% wt/vol acrylamide, 1% vol/vol glycine and 0.4% wt/vol SDS.[20] Stacking gels contained 40% vol/vol glycerol and 4% wt/vol acrylamide. After electrophoresis (275 V) for 20 h, gels were stained for 60 min in Coomassie Brilliant Blue and destained with 20% methanol and 5% acetic acid.[20] MHC bands were scanned and analyzed by the *Kodak Digital Science™ 1D Image Analyses Software*.

Statistical analysis

All values are expressed as mean ± SE. Differences among groups were determined by multivariate analysis of variance (ANOVA) with three levels for treatment—C vs. Cre7 vs. Cre14 and two levels for immobilization CL vs. I (3 × 2). Subsequently, a Tukey post hoc test was applied using Prism Graph Pad Software©. For comparison of NI and CI groups an independent *t*-test was utilized. The *P* value of confidence for statistical significance was established at 0.05.

Results

Total creatine content

After 14 days of creatine feeding, total creatine content of immobilized SOL of Cre14-I was increased by ~25% ($P<0.001$) in comparison to C-CL and C-I. Compared with C-CL, immobilized GAS of Cre14-I also responds to supplementation protocol, however, in a less pronounced manner (18%, $P<0.001$) (Table 1).

Mass parameters

Body weight after immobilization suffered a mild (but not statistically different) decrease until the 2nd day, after which it started to increase at the

Table 1 Body weight (g) and relative weight of adrenal glands (%) of NI, C, Cre7 and Cre14 groups.

	n	Body weight (g)	Weight of adrenal glands (%)
NI	6	239.8±8.1	0.032±0.003
C	6	232.7±10.1	0.038±0.004
Cre7	6	228.3±6.1	0.036±0.003
Cre14	6	238.4±6.2	0.036±0.003

Values are expressed as mean±SE. Non-immobilized group (NI; $n=6$), control group (C; $n=6$), supplemented with creatine during 7 days along with immobilization (Cre7; $n=6$) and Supplemented with creatine during 14 days (7 days before immobilization and along with immobilization) (Cre14; $n=6$).

same rate as that of the NI group (Table 1). Food intake (data not shown) in all groups accompanied the body mass change profile. No statistical differences were observed in the weight of adrenal glands among all groups (Table 2). Furthermore, no differences were observed in the wet and dry weights of the SOL and GAS muscles from the NI group, as compared to the contra-lateral leg (C-CL). The immobilization markedly decreased relative wet weight of GAS and SOL muscles in all groups ($P<0.001$) (Table 2). No statistical difference was observed between Cre7-I and C-I relative mass, however, the relative muscle mass of the SOL and GAS of Cre14-I were 21% ($P<0.05$) and 15% ($P<0.05$) greater than C-I, respectively. As observed in relative muscle mass, the dry weight, mainly composed by muscle protein, suffered a marked decrease in SOL and GAS muscles of all immobilized legs ($P<0.001$). SOL of Cre14-I demonstrated a greater dry weight in comparison to SOL of C-I (Table 3) ($P<0.05$).

Lean mass determination

The longitudinal analysis by DEXA allowed us to follow the lean mass changes over time (Fig. 1). The Δ lean mass was markedly decreased (~70%) by immobilization in all animals (CI, Cre7-I and Cre14-I; $P<0.001$). A slight lean mass loss reduction was observed in the Cre14-I as compared to CI, although this decrease was not considered statistically significant ($P<0.1$). No statistical difference in lean mass change was noted between the NI and CL of all immobilized groups.

MHC distribution

The decrease in type MHC I induced by immobilization was observed in all SOL muscles of immobilized legs (C-I, Cre7-I and Cre14-I; Fig. 2–$P<0.001$), indicating a main effect of immobilization. While the C-CL, Cre7-CL and Cre14-CL displayed more than 90% of MHC I expression, C-I, Cre7-I and Cre14-I expressed approximately 84%, 78% and 80% MHC I expression, respectively. These changes were also accompanied by an increase in MHC IIa (Fig. 3) ($P<0.001$) (see also Fig. 4). Thus, immobilization led to a shift in MHC (slow-to-fast) in the postural SOL muscle. In addition, it is noteworthy that MHC I content of Cre7-I and Cre14-I legs were reduced in comparison to C-I, thus creatine loading further enhanced the MHC I to IIa shift in the SOL muscle induced by immobilization ($P<0.05$).

Table 2 Muscle total creatine content (mmol kg^{-1} dry weight) of NI leg, C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg.

	Total creatine content		
	n	SOL	GAS
NI	6	88.7±9.0	113.3±11.4
C-CL	6	90.2±5.2	114.8±7.4
C-I	6	93.9±6.7	119.1±5.9
Cre7-CL	6	119.4±8.8^{a,b}	129.9±6.1
Cre7-I	6	104.1±6.8	122.0±7.0
Cre14-CL	6	122.3±7.2^{a,b}	138.4±9.8^{a}
Cre14-I	6	118.3±5.2^{a,b}	135.0±8.4^{a}

Values are expressed as mean±SE. Non-immobilized leg (NI; $n=6$), contra-lateral leg of the control group (C-CL; $n=6$), immobilized leg of the control group (C-I; $n=6$), contra-lateral leg of the Cre7 group (Cre7-CL; $n=6$), immobilized leg of the Cre7 group (Cre7-I; $n=6$), contra-lateral leg of Cre14 group (Cre14-CL; n=6) and immobilized leg of Cre14 group (Cre14-I; $n=6$).

[a] Different from C-CL ($P<0.001$).

[b] Different from C-I ($P<0.001$).

Discussion

In order to verify the role of creatine supplementation in skeletal muscle plasticity during disuse, we utilized a model of hind limb immobilization-induced hypokinesia. To estimate the level of stress, adrenal gland weight was determined in all immobilized groups and compared with rats not submitted to immobilization (NI). No statistical differences were observed among all groups, indicating that immobilized animals did not suffer significant additional stress. The lack of variation in weight gain and food intake indicated that the disuse model used in the present study did not interfere with developmental aspects of the animals.

Table 3 Relative muscle wet weight (muscle weight–body weight ratio) and dry muscle weight of C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg.

	Relative wet weight (%)			Dry weight (mg)		
	n	SOL	GAS	*n*	SOL	GAS
NI	12	0.50 ± 0.03	5.49 ± 0.13	12	25.8 ± 0.9	343.2 ± 14.1
C-CL	6	0.51 ± 0.02	5.44 ± 0.11	6	26.9 ± 0.7	335.3 ± 10.1
C-I	6	0.33 ± 0.02^{a}	3.91 ± 0.15^{a}	6	16.0 ± 0.8^{a}	225.7 ± 11.0^{a}
Cre7-CL	6	0.55 ± 0.01	5.57 ± 0.12	6	26.4 ± 1.5	326.9 ± 0.9
Cre7-I	6	0.38 ± 0.02^{b}	4.15 ± 0.13^{b}	6	18.5 ± 0.7^{b}	242.4 ± 12.3^{b}
Cre14-CL	6	0.53 ± 0.01	5.71 ± 0.12	6	26.2 ± 1.0	336.7 ± 18.9
Cre14-I	6	$0.42\pm0.02^{c,d}$	$4.63\pm0.13^{c,d}$	6	$19.9\pm1.5^{c,d}$	245.4 ± 19.9^{c}

Values are expressed as mean±SE. Non-immobilized leg (NI; $n=12$), contra-lateral leg of the control group (C-CL; $n=6$), immobilized leg of the control group (C-I; $n=6$), contra-lateral leg of the Cre7 group (Cre7-CL; $n=6$), immobilized leg of the Cre7 group (Cre7-I; $n=6$), contra-lateral leg of Cre14 group (Cre14-CL; $n=6$) and immobilized leg of Cre14 group (Cre14-I; $n=6$).

[a] Different from C-CL.

[b] Different from Cre7-CL ($P<0.001$).

[c] Different from Cre14-CL ($P<0.001$).

[d] Different from C-I ($P<0.05$).

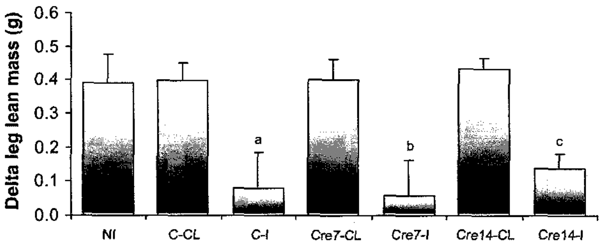

Figure 1 Leg lean mass evaluation (ΔLM) of C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-CL leg. Values are expressed as mean±SE. Contra-lateral leg of the control group (C-CL; $n=6$), immobilized leg of the control group (C-I; $n=6$), contra-lateral leg of the Cre7 group (Cre7-CL; $n=6$), immobilized leg of the Cre7 group (Cre7-I; $n=6$), contra-lateral leg of Cre14 group (Cre14-CL; $n=6$), immobilized leg Cre14 group (Cre14-I; $n=6$) and non-immobilized leg (NI; $n=6$). (ΔLM= Scan day 7–Scan day 0). (a) Different from C-CL ($P<0.001$), (b) different from Cre7-CL ($P<0.001$), (c) different from Cre14-CL ($P<0.001$).

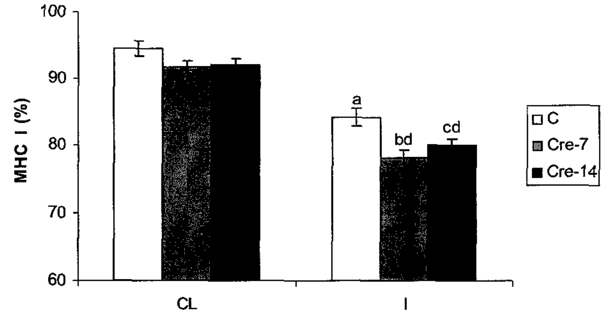

Figure 2 Myosin heavy chain I distribution of SOL from C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg. Values are expressed as mean±SE. Contra-lateral leg of the control group (C-CL; $n=6$), immobilized leg of the control group (C-I; $n=6$), contra-lateral leg of the Cre7 group (Cre7-CL; $n=6$), immobilized leg of the Cre7 group (Cre7-I; $n=6$), contra-lateral leg of Cre14 group (Cre14-CL; $n=6$) and immobilized leg Cre14 group (Cre14-I; $n=6$). (a) Different from C-CL ($P<0.001$), (b) different from Cre7-CL ($P<0.001$), (c) different from Cre14-CL ($P<0.001$), (d) different from C-I ($P<0.05$).

In order to refute the argument that the contra-lateral leg is not an adequate control reference, an age-matched group of animals that were not submitted to immobilization (NI) was utilized. Since no difference was observed in muscle mass (wet and dry) between NI and C-CL, as also demonstrated before,[16] we concluded that the contra-lateral legs are appropriate controls.

We observed, as described before,[7,21] a marked muscle mass loss (wet and dry weight) in the SOL and GAS muscles as a result of immobilization for 7 days (Table 1). In addition, we observed a marked decline in the cross sectional area of immobilized SOL fibers (type I, 26%; type IIb, 23% and type IIa, 18%; data not shown) in comparison to C-CL. This result combined with the observation of a reduction in muscle weight, confirms that the hypokinesia model utilized was effective in promoting skeletal muscle atrophy.

A recent study conducted by Hespel et al.[13] also investigated the effect of creatine supplementation during immobilization and rehabilitation in humans. These authors observed a mild decrease (~10%) in the cross-sectional area of quadriceps

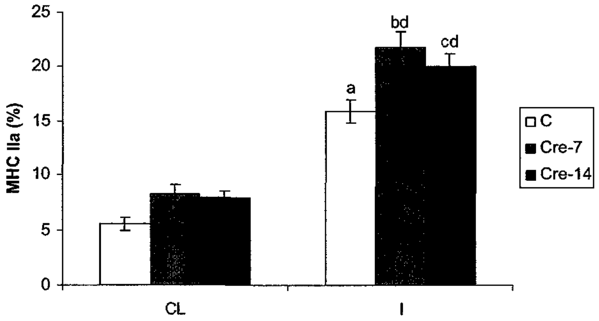

Figure 3 Myosin heavy chain IIa distribution of SOL from C-CL leg, Cre7-CL leg, Cre14-CL leg, C-I leg, Cre7-I leg and Cre14-I leg (%). Values are expressed as mean ± SE. Contra-lateral leg of the control group (C-CL; $n=6$), immobilized leg of the control group (C-I; $n=6$), contralateral leg of the Cre7 group (Cre7-CL; $n=6$), immobilized leg of the Cre7 group (Cre7-I; $n=6$), contra-lateral leg of Cre14 group (Cre14-CL; $n=6$) and immobilized leg Cre14 group (Cre14-I; $n=6$). (a) Different from C-CL ($P<0.001$), (b) different from Cre7-CL ($P<0.001$), (c) different from Cre14-CL ($P<0.001$), (d) different from C-I ($P<0.05$).

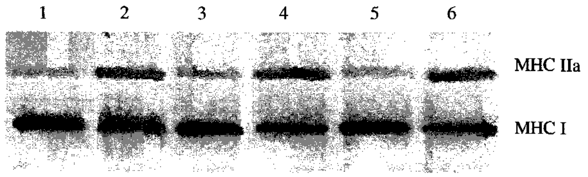

Figure 4 Representative SDS-PAGE of SOL muscle from C-CL leg, C-I leg, Cre7-CL leg, Cre7-I leg, Cre14-CL leg and Cre14-I leg. Lane 1, C-CL leg; Lane 2, C-I leg; Lane 3, Cre7-CL leg; Lane 4, Cre7-I leg; Lane 5, Cre14-CL leg and Lane 6, Cre14-I leg.

after 2 weeks of immobilization in both creatine and placebo groups, although no changes were observed in the fiber cross-sectional area after immobilization. Conversely, in the rat hypokinesia model (used in the present study), a severe loss of muscle mass and fiber cross sectional area was observed after 1 week of immobilization. It should be pointed out that, in contrast to the rat model, immobilization in humans is not performed in the totally shortened position, a condition that is well recognized to be important for accelerating the rate of muscle atrophy.[7,15] This may, at least in part, explain the apparent discrepancies between our study and the study conducted by Hespel et al.[13] who were not able to show a protective effect of creatine load upon muscle mass loss under hypokinesia.

To ensure the effectiveness of the creatine loading, we used a high dose ($5\,\mathrm{g\,kg^{-1}}$ body weight $\mathrm{day^{-1}}$). This dosage has been utilized previously and is shown to increase free creatine (30%), phosphocreatine (15%) and total creatine (20%) levels in the rat SOL in just 5 days of creatine feeding.[22] In humans, lower doses have also been found to be effective for boosting up intramuscular creatine content.[9,13,23] The supplementation protocol adopted in our study successfully increased total creatine content in SOL and whole GAS (red and white portions). As expected, SOL (mainly composed by slow-twitch fibers) showed higher creatine uptake capacity after creatine loading as compared to GAS. Op't Eijnde et al.[22] also demonstrated a significant increase only in the red portion of GAS, while the white portion of GAS showed mild increment (7%) in creatine content. Accordingly, other studies utilizing muscles which are composed by a mixture of fast and slow twitch fibers (red and white portions) such as vastus lateralis also showed an increase (20%) in creatine content after creatine loading[13].

The present study demonstrates for the first time, to our knowledge, that previous creatine loading (Cre14) minimizes skeletal muscle mass loss in a hypokinesia rat model. Creatine supplementation only during immobilization (Cre7), however, failed to promote the same response. The creatine uptake by skeletal muscle is modulated by many factors, such as the intramuscular baseline level of creatine,[23] muscle activity,[23] insulin sensitivity[24] and extracellular sodium concentration[25]. Muscle inactivity and insulin resistance observed during immobilization contributes to decrease creatine uptake capacity. This decreased creatine uptake capacity may compromise the effect of creatine supplementation upon skeletal muscle mass during immobilization (Cre7). This might explain the lack of protection against muscle mass loss observed in Cre7. It could be hypothesized that creatine loading in association to normal muscle activity is required for the attenuation of muscle wasting induced by disuse.

Our results demonstrated that the effective minimization of muscle wasting observed in the Cre14 is associated to the increment of muscle creatine content. One of the proposed mechanisms for the effect of creatine on skeletal muscle mass gain includes higher mitotic activity in satellite cells.[12] Also in line with a relationship between creatine and satellite cells, it has been recently shown that creatine activates the satellite cell differentiation program in vitro.[14] It is possible that creatine, in addition to a satellite cell mitotic effect, is also able to activate signaling pathways, protecting the skeletal muscle cell against proteolysis.

It has been reported that creatine feeding is able to increase water retention, due to its osmotic property[26] and some evidences suggest that the cell hydration state is a major factor in controlling protein turnover.[27,28] In hepatocytes, an increase in cell hydration state (swelling) acts as an anabolic signal, stimulating protein synthesis and attenuating protein breakdown. In fact, it has been already suggested that water retention induced by the increase in creatine content might function as an anabolic proliferative signal, which would involve MAPK signaling pathway that plays a pivotal role in protein systhesis regulation.[29] Although no effort has been made to address the effects of swelling in skeletal muscle, Parise et al.[30] showed that short-term creatine supplementation presented an anti-catabolic action in men, indicating a direct mechanism of creatine upon skeletal muscle protein turn over. Our results demonstrate that pre-creatine feeding (Cre14) attenuates SOL dry weight (mainly composed by muscle protein) loss, which is in line with the idea that creatine supplementation involves reduced muscle protein wasting.

The lean mass loss (indirectly determined by DEXA) in all immobilized legs confirms the results obtained from muscle weight (wet and dry). Furthermore, DEXA analysis also showed a similar muscle mass in the NI vs. C-CL groups, reinforcing that the contralateral leg might be used as a reference control. DEXA analysis also showed a tendency (although not statistically significant, $P<0.1$) of the pre-supplemented group (Cre14-I) to present a decreased muscle wasting induced by immobilization (C-I). Although DEXA analysis has great potential to be used as a non-invasive method to address loss of muscle mass,[19] our results suggest that the sensitivity of this method is not suitable to follow the effect of creatine during immobilization. This is probably due to the fact that DEXA only allows analysis of the whole leg. It is well known that muscle length during immobilization determines muscle atrophy or grow.[15] Since certain muscles (such as the *Tibialis anterior*) in the leg, in our model, are immobilized in a full stretching position (conditioning that stimulate muscle longitudinal growth), the effect of creatine supplementation is likely to be underestimated.

As previously described, hypokinesia leads to a slow-to-fast MHC shift.[31–33] These studies have shown that the proportion of MHC IIa isoform in SOL increases after hindlimb suspension. [31–33] To characterize the effect of disuse and creatine loading upon the MHC profile, we chose a postural muscle (SOL), which predominantly expresses the MHC I isoform. Our findings are in accordance with the previous data. Furthermore, in the present study, the combination of immobilization and creatine loading had an additive effect on MHC shift towards to the fast phenotype observed during immobilization. The mechanism by which creatine determines the MHC phenotype is still unknown, but it may include the modulation of intra-cellular signaling pathways involved in slow twitch phenotype determination, such as the calcineurin pathway.

The fiber phenotype was affected by creatine supplementation in immobilized SOL, however, its adaptive role remains to be elucidated. Since postural maintenance requires tonic contractile activity, the slow-to-fast shift in postural muscles, observed in disuse conditions, might represent a deleterious adaptation. Other clinical conditions such as hyperthyroidism clearly show that shift to the fast phenotype is associated with diminished muscular endurance.[34] We speculate that the increase in slow-to-fast shift observed in the immobilized SOL of creatine-fed rats could also lead to early fatigue. Functional studies should be conducted in the future to determine the role of the slow-to-fast shift driven by creatine supplementation in rats in skeletal muscle inactivity.

Since pre-treatment with creatine before immobilization attenuates the muscle wasting, we suggest that creatine supplementation might be a suitable strategy for individuals who will be submitted to hypokinesia, particularly athletes in the pre-operative phase involved in osteo-articular injuries. In such conditions, to ensure surgery success, which is followed by a prolonged post-operative immobilization, an intense muscle re-inforcement program should be initiated prior to surgery. Therefore, creatine supplementation during the muscle reinforcement program might be a viable dietary manipulation to maximize muscle mass gain before surgery and also minimize muscle wasting during immobilization.

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Letter to the Editor

To the Editor:

Two recent articles reviewed the use of oral creatine to enhance athletic performance and its potential side effects (Juhn MS, Tarnopolsky M. *Clin J Sport Med 1998; 8:286–297 and 298–304).* In their review of safety concerns, however, the authors do not mention that under conditions that exist in the human stomach (high acidity, supply of nitrite from food and saliva), creatine can react to form N-nitrososarcosine.[1] N-Nitrososarcosine has been shown to induce esophageal cancer in rats[2] and liver cancer in mice.[3] Formation of N-nitrososarcosine is much more likely to occur after ingestion of pure creatine as a "drug" rather than as a component of food, because foods that naturally contain creatine, such as meat, contain large amounts of other nitrogenous components with which gastric nitrous acid can react to form innocuous products. Although N-nitrososarcosine is a relatively weak carcinogen and although it has not been shown directly that it forms in the human stomach, the potential for the formation of this carcinogenic compound, the effects of which would only become apparent after many years, warrants consideration by those advocating the safety of creatine as a performance-enhancing substance.

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Author Reply

We would like to thank Dr. Archer for his comments on our recent articles, examining performance and safety issues related to oral creatine supplementation. First, it should be noted that neither of the authors "advocate the safety of creatine as a performance-enhancing substance," but rather present it and evaluate the available literature on both athletic performance and side effects. We were unaware of this interesting work recorded by Dr. Archer and colleagues, and believe it is important that this be brought forth in this medium.

Dr. Archer appropriately points out that there are several steps required in the evaluation of a potential link between oral creatine supplementation, the formation of N-nitrososarcosine, and cancer in humans. In one of their studies,[1] when 5.2 g of creatine was added to 25% hydrochloric acid and 5 g of sodium nitrate, N-nitrososarcosine was formed after reacting for 2.5 hours. Clearly, this amount of nitrite would not likely be found in the stomach in the absence of a concomitant meal. As the author points out, nitrogen-rich foods also contain other nitrogenous compounds, which are likely to form innocuous products.

In a separate publication, the authors demonstrated that intraperitoneal injection of nitrososarcosine at 225 mg/kg induced liver cancer in approximately 50% of animals. If one uses the *in vitro* calculations for N-nitrososarcosine formation from the original study, approximately 1 g of nitrososarcosine can be formed from 5 g of creatine under ideal conditions. Therefore, this would amount to 14 mg/kg, or one sixteenth of the dose used in the carcinogenesis experiments. In animal feeding studies with creatine doses greater than those possible in humans, there was an observed inhibition of sarcoma and mammary tumors in rats after creatine administration.

The comments raised by Dr. Archer do not demonstrate a direct link between oral creatine supplementation and carcinogenesis in humans, yet certainly raise the possibility of an intriguing biochemical pathway link, which should be a further warning to athletes that overconsumption of creatine—and indeed many other compounds, including many food products—may have deleterious long-term effects. What is needed at this time are studies of N-nitrososarcosine production in the human stomach in response to various doses of creatine and life-long studies of creatine administration in rodents at doses expected in athletes. Detailed pathologic studies also should be performed to determine whether there is any increase in carcinogenesis or other potential side effects.

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Letter to the Editor

Creatine: a safety concern

The safety of creatine to enhance athletic performance was recently reviewed (Brudnal, 2004) without reference to a potentially important toxicological problem. Under conditions that exist the human stomach (high acidity and supply of nitrite from food and saliva), creatine can react to form *N*-nitrososarcosine (Archer et al., 1971) that is known to induce esophageal cancer in rats (Druckrey et al., 1967) and stomach cancer in mice (Wogan et al., 1975). As I have previously suggested (Archer, 1999), the potential for the formation of this carcinogen should be considered by those advocating the safety of creatine as a performance-enhancing substance.

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Letter to the Editor

To the Editor: The use of creatine to enhance athletic performance was recently reviewed in *Nutrition*[1] without mention of a potentially important safety concern. Creatine can react to form N-nitrososarcosine[2] under conditions that exist the human stomach (high acidity and supply of nitrite from food and saliva). N-Nitrososarcosine is known to induce esophageal cancer in rats[3] and stomach cancer in mice.[4] As I have suggested in response to other reviews of creatine,[5,6] the possible formation of this carcinogen should be considered by those advocating the safety of creatine as a performance-enhancing substance, particularly when relatively large amounts may be consumed in the form of supplements.

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Dietary nitrate supplementation reduces the O_2 cost of low-intensity exercise and enhances tolerance to high-intensity exercise in humans

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Abstract

Pharmacological sodium nitrate supplementation has been reported to reduce the O_2 cost of sub-maximal exercise in humans. In this study, we hypothesised that dietary supplementation with inorganic nitrate in the form of beetroot juice (BR) would reduce the O_2 cost of sub-maximal exercise and enhance the tolerance to high-intensity exercise. In a double-blind, placebo-controlled, crossover study, eight males (aged 19-38 yr) consumed 500 mL per day of either beetroot juice (BR, containing 11.2 ± 0.6 mM of nitrate) or blackcurrant cordial (as a placebo, PL, with negligible nitrate content) for six consecutive days, and completed a series of 'step' moderate-intensity and severe-intensity exercise tests on the last 3 days. On days 4-6, plasma [nitrite] was significantly greater following dietary nitrate supplementation compared to placebo (BR: 273 ± 44 *vs*. PL: 140 ± 50 nM; $P<0.05$) and systolic blood pressure was significantly reduced (BR: 124 ± 2 *vs*. PL: 132 ± 5 mmHg; $P<0.01$). During moderate exercise, nitrate supplementation reduced muscle fractional O_2 extraction (as estimated using near infra-red spectroscopy). The gain of the increase in pulmonary $\dot{V}O_2$ following the onset of moderate exercise was reduced by 19% in the BR condition (BR: 8.6 ± 0.7 *vs*. PL: 10.8 ± 1.6 $mL \cdot min^{-1} \cdot W^{-1}$; $P<0.05$). During severe exercise, the $\dot{V}O_2$ slow component was reduced (BR: 0.57 ± 0.20 *vs*. PL: 0.74 ± 0.24 L.min-1; $P<0.05$) and the time-to-exhaustion was extended (BR: 675 ± 203 *vs*. PL: 583 ± 145 s; $P<0.05$). The reduced O_2 cost of exercise following increased dietary nitrate intake has important implications for our understanding of the factors which regulate mitochondrial respiration and muscle contractile energetics in humans.

Running Head: Dietary nitrate and $\dot{V}O_2$ kinetics

Key Words: exercise economy, muscle efficiency, O_2 uptake, exercise performance, $\dot{V}O_2$ kinetics, fatigue

Introduction

A fundamental tenet of human exercise physiology is a predictable oxygen (O_2) cost for a given sub-maximal work rate. Upon the initiation of moderate-intensity exercise (i.e. exercise performed at work rates below the gas exchange threshold, GET), pulmonary O_2 uptake ($\dot{V}O_2$), which closely reflects O_2 consumption in the skeletal muscles (2, 28, 38), rises in an exponential fashion to attain a 'steady-state' within approximately 2-3 min in healthy humans (64). The steady-state increase in $\dot{V}O_2$ is linearly related to the increase in external work rate; is essentially independent of factors such as age, health status or aerobic fitness; and approximates 10 mL O_2 per minute per Watt of external power output during cycle ergometry (i.e., $10\ mL{\cdot}min^{-1}{\cdot}W^{-1}$; 36). During supra-GET exercise, $\dot{V}O_2$ dynamics become more complex owing, in part, to the development of a delayed-onset $\dot{V}O_2$ 'slow component' which elevates the O_2 cost of exercise above $10mL{\cdot}min^{-1}{\cdot}W^{-1}$ (36, 64).

Whereas it is known that interventions such as training and the inspiration of hyperoxic gas can reduce the O_2 cost of heavy (above the GET but below critical power, CP, 52) and severe (>CP) exercise by reducing the amplitude of the $\dot{V}O_2$ slow component, the steady-state $\dot{V}O_2$ during moderate exercise is unaffected by these and other interventions in healthy humans (1, 15, 36, 51, 65). Surprisingly, however, it was recently reported that 6 days of dietary supplementation with pharmacological sodium nitrate reduced the O_2 cost of sub-maximal cycling at work rates expected to require 45-80% $\dot{V}O_2$ max (45). That this effect occurred without any increase in estimated non-oxidative energy production (as reflected by an unchanged blood [lactate]) suggested that sodium nitrate ingestion improved the efficiency of muscle oxidative metabolism. It is known that tolerance to high-intensity exercise is, in certain respects, a function of $\dot{V}O_2$ max and sub-maximal exercise economy (20). Therefore, assuming that $\dot{V}O_2$ max is not altered by dietary nitrate, it is feasible that dietary nitrate supplementation might enhance exercise tolerance. However, this possibility has not been investigated.

The nitrate anion (NO_3^-) is relatively inert, and thus any biological effects are likely conferred via its conversion to the bioactive nitrite anion (NO_2^-). Inorganic nitrate is rapidly absorbed from the gut and is concentrated in saliva at least 10-fold. In the mouth, facultative anaerobic bacteria on the surface of the tongue reduce NO_3^- to NO_2^- (23). Nitrite can be converted to nitric oxide (NO) in the stomach (6, 47) but it is also clear that some is absorbed to increase circulating plasma [nitrite] (21, 46). We and several other groups have shown that NO_2^- can be converted to NO under appropriate physiological conditions (6). The requisite one electron reduction has variously been reported to be catalysed via xanthine oxidoreductase, haemoglobin, myoglobin, endothelial nitric oxide synthase, and the mitochondrial electron transfer complexes (61).

There are at least two mechanisms by which NO derived from NO_2^- (rather than from the much better known synthesis of NO from L-arginine by the NO synthase family of enzymes) might influence O_2 utilization by contracting skeletal muscle. Firstly, as all of the known mechanisms for NO_2^- reduction are facilitated by hypoxia, it may be that more NO (which is a potent vasodilator) is generated in parts of muscle which are receiving less or using more O_2, and therefore this mechanism would help to match local blood flow to O_2 requirement, providing a more homogenous distribution of O_2 within skeletal muscle. However, while this might be beneficial in terms of muscle function, it would not explain a reduced O_2 cost during exercise. A second possible mechanism involves the roles of NO_2^- and NO as regulators of cellular O_2 utilization. For example NO is known to be an important inhibitor of cytochrome oxidase activity (10). More recently, it has been suggested that NO might enhance the efficiency of oxidative phosphorylation by reducing 'slippage' of the mitochondrial proton pumps (17). There is also evidence that NO_2^- can serve as an alternative electron acceptor, theoretically replacing the role of O_2 in respiration (3).

The diet constitutes the main source of NO_3^- in humans, with vegetables accounting for 60-80% of daily NO_3^- intake in a Western diet (67). Given the reported ability of pharmacological sodium nitrate to reduce the O_2 cost of exercise (45), we sought to determine whether similar effects are observed when the NO_3^- dose is administered in the form of nitrate-rich beetroot juice (BR). This is important because sodium nitrate is a pharmaceutical product whereas BR is a natural food product that can be readily ingested as part of the normal diet. We therefore investigated the influence of BR ingestion on plasma [nitrite], blood pressure (BP), muscle oxygenation (assessed with near infra-red spectroscopy, NIRS) and the $\dot{V}O_2$ response to step transitions to moderate and severe intensity exercise. We hypothesized that dietary BR supplementation would reduce the O_2 cost of moderate-intensity exercise and increase exercise tolerance (assessed as the time to task failure) during severe-intensity exercise.

Methods

Subjects

Eight healthy males (mean ± SD, age 26 ± 7 yr, height 180 ± 3 cm, body mass 82 ± 6 kg; maximal O_2 uptake ($\dot{V}O_2$ max) 49 ± 5 $mL \cdot kg^{-1} \cdot min^{-1}$) who were recreationally active in sporting activities volunteered to participate in this study. None of the subjects were tobacco smokers or users of dietary supplements. All subjects were fully familiar with laboratory exercise testing procedures, having previously participated in studies employing cycle ergometry in our laboratory. The procedures employed in this study were approved by the Institutional Research Ethics Committee. All subjects gave their written informed consent prior to the commencement of the study, after the experimental procedures, associated risks, and potential benefits of participation had been explained. Subjects were instructed to arrive at the laboratory in a rested and fully hydrated state, at least 3 h postprandial, and to avoid strenuous exercise in the 24 h preceding each testing session. Each subject was also asked to refrain from caffeine and alcohol intake 6 and 24 h before each test, respectively. All tests were performed at the same time of day (± 2 hours).

Procedures

The subjects were required to report to the laboratory on seven occasions, over a 4-5 week period. During the first visit to the laboratory, subjects performed a ramp incremental exercise test for determination of the $\dot{V}O_{2peak}$ and gas exchange threshold (GET). All cycle tests were performed on an electronically braked cycle ergometer (Lode Excalibur Sport, Groningen, the Netherlands). Initially, subjects completed 3 min of 'unloaded' baseline cycling, after which the work rate was increased at a rate of 30 $W \cdot min^{-1}$ until the subject was unable to continue. The participants cycled at a self-selected pedal rate (70-90 rpm), and this pedal rate along with saddle and handle bar height and configuration were recorded and reproduced in subsequent tests. The breath-by-breath pulmonary gas-exchange data were collected continuously during the incremental tests and averaged over consecutive 10-s periods. The $\dot{V}O_{2peak}$ was taken as the highest 30-s average value attained prior to the subject's volitional exhaustion. The GET was determined as described previously (1, 5). The work rates that would require 80% of the GET (moderate exercise) and 70% Δ (70% of the difference between the power output at the GET and $\dot{V}O_{2\ peak}$, severe exercise) were subsequently calculated with account taken of the mean response time for $\dot{V}O_2$ during ramp exercise (i.e., two thirds of the ramp rate was deducted from the power output at GET and peak; 63).

Following completion of the ramp test, subjects were randomly assigned in a cross-over design, to receive 6 days of dietary supplementation with either nitrate (NO_3^-; 5.5 mmol·day^{-1}; administered as 0.5 L organic beetroot juice per day^{-1}; Beet It, James White Drinks Ltd., Ipswich) or "placebo" (low-calorie blackcurrant juice cordial with negligible nitrate content). The subjects were instructed to sip the beverages at regular intervals throughout the day. A 10 day washout separated the supplementation periods. The order between the nitrate and placebo supplementation periods was balanced. The subjects were provided with a list of foods rich in nitrates and asked to abstain from consuming these foods for the duration of the study. The subjects were not aware of the experimental hypotheses to be tested but were informed that the purpose of the study was to compare the physiological responses to exercise following the consumption of two commercially available beverages. The personnel administering the exercise tests were not aware of the type of beverage being consumed by the subjects.

On days 4, 5 and 6 of the supplementation periods, the subjects completed 'step' exercise tests from a 20 W baseline to moderate and severe intensity work rates for the determination of pulmonary $\dot{V}O_2$ dynamics. On the fourth day of supplementation, subjects completed two bouts of moderate cycling, while on days five and six the subjects completed one bout of moderate cycling and one bout of severe cycling. The two bouts of exercise on each day were separated by 25 min of passive recovery. All exercise bouts were of 6 min duration, with the exception of the severe exercise bout on the final day which was continued until task failure as a measure of exercise tolerance. The time to task failure was recorded when the pedal rate fell by >10 rpm below the self-selected pedal rate. In these bouts, the subjects were verbally encouraged to continue for as long as possible. The $\dot{V}o_2$ responses to the four moderate and two severe exercise bouts were averaged prior to analysis to reduce breath-to-breath noise and enhance confidence in the parameters derived from the modeling process (44). Before each exercise bout, blood pressure was measured and venous blood samples were collected for subsequent determination of plasma [nitrite] (see 'Measurements').

Measurements

During all tests, pulmonary gas exchange and ventilation were measured continuously using a portable metabolic cart (MetaMax 3B, Cortex Biophysik, Leipzig, Germany), as described previously (1). A turbine digital transducer measured inspired and expired airflow while an electro-chemical cell O_2 analyzer and an infrared CO_2 analyzer simultaneously measured expired gases. Subjects wore a nose clip and breathed through a low dead-space, low-resistance mouthpiece that was securely attached to the volume transducer. The inspired and expired gas volume and gas concentration signals were continuously sampled via a capillary line connected to the mouthpiece and displayed breath-by-breath. Heart rate (HR) was measured during all tests using short-range radiotelemetry (Polar S610, Polar Electro Oy, Kempele, Finland). During one of the transitions to moderate and severe exercise, for both supplementation periods, a blood sample was collected from a fingertip into a capillary tube over the 20 s preceding the step transition in work rate and within the last 20 s of exercise. A capillary blood sample was also collected at the limit of tolerance for the severe bout performed on day 6 of each supplementation period. These whole blood samples were subsequently analyzed to determine blood lactate concentration ([lactate]) (YSI 1500, Yellow Springs Instruments, Yellow Springs, OH) within 30 s of collection. Blood lactate accumulation (Δ blood [lactate]) was calculated as the difference between blood [lactate] at end-exercise and blood [lactate] at baseline.

The oxygenation status of the *m. vastus lateralis* of the right leg was monitored using a commercially available NIRS system (model NIRO 300, Hamamatsu Photonics KK, Hiugashiku, Japan). The system consisted of an emission probe that irradiates laser beams and a detection probe. Four different wavelength laser diodes provided the light source (776, 826, 845, and 905 nm), and the light returning from the tissue was detected by a photomultiplier tube in the spectrometer. The intensity of incident and transmitted light was recorded continuously at 2 Hz and used to estimate concentration changes from the resting baseline for oxygenated, deoxygenated, and total tissue hemoglobin/myoglobin. Therefore, the NIRS data represent a relative change based on the optical density measured in the first datum collected. The [HHb] signal can be regarded as being essentially blood-volume insensitive during exercise and so was assumed to reflect the balance between local O_2 supply and utilization and to provide an estimate of changes in O_2 extraction in the field of interrogation (1, 22, 24, 30). The leg was initially cleaned and shaved around the belly of the muscle, and the probes were placed in the holder, which was secured to the skin with adhesive at 20 cm above the fibular head. To secure the holder and wires in place, an elastic bandage was wrapped around the subject's leg. The wrap helped to minimize the possibility that extraneous light could influence the signal and also ensured that the optodes did not move during exercise. Pen marks were made around the probes to enable precise reproduction of the placement in subsequent tests. The probe gain was set with the subject at rest in a seated position with the leg extended at down stroke on the cycle ergometer before the first exercise bout, and NIRS data were collected continuously throughout the exercise protocols. The data were subsequently downloaded onto a personal computer, and the resulting text files were stored on disk for later analysis.

Blood pressure of the brachial artery was measured with subjects in a rested, seated position prior to each exercise bout using an automated sphygmomanometer. Three measurements were taken at each sample point with the mean of the second and third blood pressure measurements being recorded. Venous blood samples were also drawn into lithium-heparin tubes prior to each exercise bout and centrifuged at 4000 rpm and 4°C for 10 min, within 3 min of collection. Plasma was subsequently extracted and immediately frozen at -80°C, for later analysis of nitrite (NO_2^-) via chemiluminescence (4).

All glass wear, utensils and surfaces were rinsed with deionized water to remove residual NO_2^- prior to analysis. After thawing at room temperature, plasma samples were initially deproteinised prior to analysis using the procedures of Higuchi and Motomizu (33). Initially, 100 μl of sample was placed in a microcentrifuge tube along with 200 μl of deionised H_2O and 300 μl 0.3 N NaOH, and left to stand at room temperature for 5 min. Then, 300 μl of 5% by weight aqueous $ZnSO_4$ was added to the mixture, after which the sample was vortexed and left to stand at room temperature for a further 10 min. Thereafter, samples were centrifuged at 4000 rpm for 15 min and the supernatant was removed for subsequent analysis. The $[NO_2^-]$ of the deproteinised plasma samples was determined by its reduction to NO in the presence of 5ml glacial acetic acid and 1% NaI under nitrogen at room temperature in a gas-sealed purging vessel. Samples were introduced to the vessel via injection into the septum at the top of the vessel. The NO content was quantified by a chemiluminescence nitric oxide analyzer (Sievers, 280i nitric oxide analyzer, CO, USA). The reaction of NO with ozone in the chemiluminescent reaction chamber yielded electronically excited NO_2 (nitrogen dioxide) which emits light at the infra-red region of the electromagnetic spectrum. Ozone was generated from an O_2 supply via an electrostatic ozone generator and high voltage transformer. To minimize the interference of the chemiluminescent reactions of sulfur-containing compounds an optical filter transmitted only red wavelengths (>600 nm), since the light emitted by sulfur-containing compounds is of shorter wavelengths. The intensity of the filtered infra-red light was quantified by a red-sensitive photomultiplier tube and amplified producing an analog mV output signal. The $[NO_2^-]$ was derived from the integral of the NO-generated mV signal over time compared to those obtained for $NaNO_2^-$ standards.

Data Analysis Procedures

The breath-by-breath $\dot{V}O_2$ data from each test were initially examined to exclude errant breaths caused by coughing, swallowing, sighing, etc., and those values lying more than four standard deviations from the local mean were removed. The breath-by-breath data were subsequently linearly interpolated to provide second-by-second values and, for each individual, identical repetitions were time-aligned to the start of exercise and ensemble-averaged. The two severe exercise bouts were of different duration (6 min for the first bout and > 6 min in the second bout which was performed to task failure) and so, at this intensity, only the first 6 min of data were averaged together and modeled. The first 20 s of data after the onset of exercise (i.e., the phase I response) were deleted and a nonlinear least-square algorithm was used to fit the data thereafter. A single-exponential model was used to characterize the $\dot{V}O_2$ responses to moderate exercise and a bi-exponential model was used for severe exercise, as described in the following equations:

$$\dot{V}O_2\,(t) = \dot{V}O_{2\text{ baseline}} + A_p\,(1\text{-}e^{-(t\text{-}TDp/\tau p)}) \qquad \text{(moderate)} \qquad \text{(Eqn. 1)}$$

$$\dot{V}O_2\,(t) = \dot{V}O_{2\text{ baseline}} + A_p\,(1\text{-}e^{-(t\text{-}TDp/\tau p)}) + A_s\,\,(1\text{-}e^{-(t\text{-}TDs/\tau s)}) \qquad \text{(severe)} \qquad \text{(Eqn. 2)}$$

where $\dot{V}O_2\,(t)$ represents the absolute $\dot{V}O_2$ at a given time t; $\dot{V}O_{2\text{baseline}}$ represents the mean $\dot{V}O_2$ in the baseline period; A_p, TD_p, and τ_p represent the amplitude, time delay, and time constant, respectively, describing the phase II increase in $\dot{V}O_2$ above baseline; and A_s, TD_s, and τ_s represent the amplitude of, time delay before the onset of, and time constant describing the development of, the $\dot{V}O_2$ slow component, respectively.

An iterative process was used to minimize the sum of the squared errors between the fitted function and the observed values. $\dot{V}O_{2\text{baseline}}$ was defined as the mean $\dot{V}O_2$ measured over the final 90 seconds of baseline pedaling. The end-exercise $\dot{V}O_2$ was defined as the mean $\dot{V}O_2$ measured over the final 30 seconds of exercise. Because the asymptotic value (A_s) of the exponential term describing the $\dot{V}O_2$ slow component may represent a higher value than is actually reached at the end of the exercise, the actual amplitude of the $\dot{V}O_2$ slow component at the end of exercise was defined as A_s'. The A_s' parameter was compared at the same iso-time (360-s) under both supplementation periods. The amplitude of the slow component was also described relative to the entire $\dot{V}O_2$ response. In addition, the functional 'gain' (G) of the fundamental $\dot{V}O_2$ response was computed by dividing A_p by the Δ work rate. The functional gain of the entire response (i.e., end-exercise 'gain') was calculated in a similar manner. To determine the overall kinetics of the $\dot{V}O_2$ response to both moderate- and severe-intensity exercise, data were fit with a mono-exponential model from 0-s to end-exercise, without time delay.

To provide information on muscle oxygenation, we also modeled the [HHb] response to exercise. Mono- and biexponential models, similar to those described above, were applied to the ensemble averaged data with the exception that the fitting window commenced at the time at which the [HHb] signal increased 1 SD above the baseline mean (22). The [HHb] kinetics for moderate exercise were determined by constraining the fitting window to the point at which mono-exponentiality became distorted, consequent to a gradual fall in [HHb] (1), as determined by visual inspection of the residual plots. The [HHb] kinetics for severe exercise were determined by fitting a bi-exponential model from the first data point, which was 1 SD above the baseline mean through the entire response. The [HHb] TD and τ values were summed to provide information on the overall [HHb] response dynamics in the fundamental phase of the response. The [HbO_2] and [Hbtot] responses do not approximate an exponential (22) and were, therefore, not modeled. Rather, we assessed changes in these parameters by determining the [HbO2] and [Hbtot] at baseline (90-s preceding step transition), 120 s, and end exercise (average response over the final 30 s of exercise).

We also modeled the HR response to exercise in each condition. For this analysis, HR data were linearly interpolated to provide second-by-second values, and, for each individual, identical repetitions from like transitions were time aligned to the start of exercise and ensemble averaged. Nonlinear least squares mono-exponential and bi-exponential models without TD were used to fit the data to moderate and severe-intensity exercise, respectively, with the fitting window commencing at $t = 0$ s. The HR τ_p so derived provides information on the overall HR response dynamics.

Statistics

Differences in the cardiorespiratory and NIRS-derived variables between conditions were analyzed with two-tailed, paired-samples *t*-tests. Alterations in blood pressure and plasma [NO_2^-] were determined via a two-tailed, two-way (supplement × time) repeated-measures ANOVA. Significant effects were further explored using simple contrasts with the alpha level adjusted via a Bonferroni correction. Correlations were assessed via Pearson's product moment correlation coefficient. Data are presented as mean ± SD, unless otherwise stated. Statistical significance was accepted when $P<0.05$.

Results

The BR supplementation regimen implemented in this study was well tolerated with no deleterious side effects. Subjects did, however, report beeturia (red urine) and red stools, consistent with a previous study (62).

Plasma [NO_2^-] and blood pressure

The group mean plasma [NO_2^-] values obtained at the two sample points on each of days 4, 5 and 6 of the BR and PL supplementation periods are illustrated in Fig 1. Participants showed elevations in plasma [NO_2^-] during the BR supplementation compared to PL at all sample points (Fig 1). On average, across the six sample points, BR ingestion increased plasma [NO_2^-] by 96%. The BR-induced elevations in plasma [NO_2^-] were not different across days 4-6.

The group mean systolic blood pressure values measured at the six BR and PL sample points are shown in Fig 2. The ingestion of BR significantly reduced systolic BP at five of the six sample points, relative to placebo (Table 1). Overall, systolic blood pressure was reduced by 6 mmHg across the six samples points (Fig 2); however, similar to plasma [NO_2^-], the BR-induced reductions in systolic BP were not significantly different among days 4-6. The systolic BP was significantly related to the plasma [NO_2^-] on day 5 ($r = -0.71$, $P < 0.05$) but no relationships were detected between systolic BP and plasma [NO_2^-] on days 4 or 6. Diastolic BP (~ 72 ± 8 mmHg) and mean arterial pressure (~ 91 ± 5 mmHg) were not significantly affected by BR ingestion.

NIRS measurements

Moderate exercise

The [O_2Hb], [HHb] and [Hbtot] values measured during moderate exercise are shown in Table 1 and the group mean responses are shown in Fig 3. A 13% reduction in the [HHb] amplitude was observed following BR ingestion, indicating that fractional O_2 extraction was reduced (PL: 88 ± 38 *vs.* BR: 78 ± 34 AU; $P<0.05$; Fig 3). The [O_2Hb] within the microvasculature was increased at baseline and at 2 min into exercise, but was not significantly different at the end of exercise (Table 1). BR ingestion elevated [Hbtot] (an index of vascular red blood cell content) at baseline; however, this effect was not maintained during exercise (Table 1).

Severe exercise

The [O_2Hb], [HHb] and [Hbtot] values measured during severe exercise are shown in Table 1 and the group mean response is shown in Fig 4. In contrast to the BR-induced changes in indices of muscle oxygenation during moderate exercise, the [HHb], [O_2Hb], and [Hbtot] parameters were unaffected by BR ingestion during severe exercise.

Oxygen uptake dynamics and exercise tolerance

Moderate exercise

The pulmonary $\dot{V}\text{O}_2$ response during moderate exercise is highlighted in Fig 5 and the parameters derived from the model fit are presented in Table 2. Dietary BR supplementation resulted in a 19% reduction in the amplitude of the pulmonary $\dot{V}\text{O}_2$ response, relative to PL, following a step increment to the same absolute moderate-intensity cycling work rate (Pl: 640 ± 146 *vs.* BR: 521 ± 153 ml·min^{-1}; $P<0.01$; Fig 5), with there being no difference in $\dot{V}\text{O}_2$ during the baseline period of very low intensity (20 W) cycling. Accordingly, the functional 'gain' (i.e., the ratio of the increase in O_2 consumed per minute to the increase of external power output) was reduced from 10.8 mL·min^{-1}·W^{-1} following PL supplementation to 8.6 mL·min^{-1}·W^{-1} following BR supplementation. The absolute $\dot{V}\text{O}_2$ value over the final 30-s of moderate exercise was also significantly lower following BR ingestion (PL: 1517 ± 123 *vs.* BR: 1448 ±

129 ml·min^{-1}; $P<0.01$; Fig 5). The phase II time constant was not significantly altered by BR supplementation (PL: 26 ± 7 *vs.* BR: 29 ± 6 s; $P>0.05$). The 95% confidence interval for the estimation of the phase II time constant was 3 ± 1 s for both conditions. The baseline and end-exercise values of $\dot{V}_{CO_2}$, $\dot{V}_E$, respiratory exchange ratio (RER), HR and blood [lactate] were not significantly between the conditions (Tables 2 and 3).

Severe exercise

The pulmonary $\dot{V}_{O_2}$ response during severe exercise is shown in Fig 6 and the parameters derived from the bi-exponential fit are presented in Table 2. In contrast to the effects observed for moderate exercise, the primary $\dot{V}_{O_2}$ amplitude during severe exercise was significantly elevated following BR supplementation (PL: 2158 ± 168 *vs.* BR: 2345 ± 179 ml·min^{-1}; $P<0.05$). Additionally, the phase II time constant was significantly greater following BR supplementation relative to placebo (PL: 33 ± 11 *vs.* BR: 40 ± 13 s; $P<0.05$; Table 2). The 95% confidence intervals for the estimation of the phase II time constant were 5 ± 2 s and 6 ± 3 s for the PL and BR conditions, respectively. The amplitude of the $\dot{V}_{O_2}$ slow component was significantly smaller following BR supplementation (PL: 739 ± 242 *vs.* BR: 568 ± 195 ml·min^{-1}; $P<0.05$), and therefore represented a smaller proportion of the overall $\dot{V}_{O_2}$ response (PL: 25 ± 6 *vs.* BR: 19 ± 6 %; $P<0.05$). The $\dot{V}_{O_2}$ attained at task failure was not different either between the conditions or from the $\dot{V}_{O_2}$ max recorded during the initial ramp incremental test. Exercise tolerance was enhanced following BR supplementation as demonstrated by the increased time to task failure (PL: 583 ± 145 *vs.* BR: 675 ± 203 s; $P<0.05$; Table 2). However, the increased time to task failure was not correlated with the reduction of the $\dot{V}_{O_2}$ slow component (r = -0.14; P = 0.70). The baseline and end-exercise values of $\dot{V}_{CO_2}$, $\dot{V}_E$, RER and HR were not significantly between the conditions (Tables 2 and 3). Blood [lactate] at 6 min of exercise and at task failure was also not significantly different between the conditions (Table 3).

Discussion

The principal original finding of this investigation is that three days of dietary supplementation with nitrate-rich beetroot juice (which doubled the plasma [nitrite]) significantly reduced the O_2 cost of cycling at a fixed sub-maximal work rate and increased the time to task failure during severe exercise. These findings were consistent with our experimental hypotheses. The O_2 cost of cycling at a fixed moderate work rate is known to be highly consistent in human populations irrespective of factors such as age and training status (36, 49). That an acute nutritional intervention (i.e., dietary supplementation with a natural food product that is rich in nitrate) can reduce the O_2 cost of a given increment in work rate by ~20% is therefore remarkable.

Short-term (i.e. 4-6 days) dietary supplementation with BR increased plasma [NO_2^-] by ~96% in this investigation. Consistent with these findings, dietary NO_3^- supplementation has previously been shown to increase plasma [NO_2^-] when administered as either sodium nitrate (45) or BR (62). Importantly, interrupting the entero-salivary circulation by spitting out saliva thwarted the rise in plasma [NO_2^-] (62), while administration of antibacterial mouthwash prior to sodium nitrate ingestion also prevented the rise in plasma [NO_2^-] by decreasing the NO_3^- reducing bacteria counts in the oral cavity (29). Collectively, these data highlight the dependence of the NO_3^- to NO_2^- conversion pathway on the commensal bacterial nitrate reductases present in the human oral cavity. The bacterially-derived NO_2^- can increase circulating plasma NO_2^- and undergo reduction to yield NO in hypoxia or acidosis (21, 46).

Effects of dietary nitrate on blood pressure

In the present study, dietary supplementation with BR reduced systolic BP by an average of 6 mm Hg, but without altering diastolic BP or MAP. In contrast, both systolic and diastolic BP was reduced following 3 days of dietary NO_3^- supplementation in the study of Larsen et al. (45). Recent data indicate that, following BR ingestion, peak reductions in systolic and diastolic BP are observed 2.5 and 3 hrs post-ingestion, respectively (62). Furthermore, the BR-induced reduction in systolic BP persisted for 24 hrs post-ingestion, while diastolic BP had returned towards baseline (62). Collectively, these data suggest that systolic BP is more amenable to nitrate-induced change than is diastolic BP. The reduced BP observed with a diet rich in nitrates suggests that this 'natural' approach has the potential to maintain or enhance aspects of cardiovascular health.

The NO_3^--induced reduction in systolic BP is likely mediated via its conversion to NO_2^-, and thence NO. Nitric oxide is known to be an important endothelial relaxing factor, through its role as a secondary messenger in cyclic guanosine mono-phosphate (cGMP) synthesis, culminating in smooth muscle relaxation (31). Conventionally, NO production was considered to be derived, universally, via the NO synthase family of enzymes in humans. These enzymes produce NO through catalyzing the five electron oxidation of L-arginine in a reaction requiring oxygen (O_2) and NADPH (9). More recent research has identified an O_2-independent pathway for the generation of NO, via the reduction of nitrite (NO_2^-) to NO in an acidic milieu (6). Importantly, this pathway preserves blood and tissue NO production during hypoxia when the enzymatic activity of the NO synthases are rate-limited by the lack of O_2 availability. As such, a considerable body of evidence now supports a biological role for NO_2^- in hypoxic vasodilation (26) which serves to protect tissues from ischemia and reperfusion injury (27).

Effects of dietary nitrate on the physiological responses to moderate exercise

Increased dietary NO_3^- consumption altered indices of muscle oxygenation as investigated using NIRS. During moderate exercise, [Hbtot] was elevated at baseline and [O_2Hb] was elevated both at baseline and over the first 120 s of exercise following BR ingestion. The increased blood volume in the region of interrogation at baseline following BR ingestion is presumably a consequence of enhanced muscle vasodilatation resulting from increased NO production from NO_2^-. The NIRS-derived [HHb] response reflects the balance between local O_2 delivery and utilization and has been used previously as an index of muscle fractional O_2 extraction (1, 22, 24, 30). In the present study, the amplitude of the [HHb] response was reduced by 13% following BR ingestion. Conversely, inhibition of NO synthesis via L-NAME administration has been shown to increase muscle O_2 extraction in the exercising horse (40). By the Fick equation, for the same $\dot{V}O_2$, an increased muscle O_2 delivery would be expected to enable a reduced muscle fractional O_2 extraction. However, in the present study in which $\dot{V}O_2$ was reduced by dietary BR supplementation, an alternative interpretation is that less O_2 extraction was required consequent to a reduced aerobic energy turnover or muscle energy utilization.

Perhaps the most striking finding of the present investigation was the significant reduction in the O_2 cost of sub-maximal exercise following increased dietary NO_3^- intake. While the $\dot{V}O_2$ response during the unloaded baseline cycle was unaffected, a 19% reduction in the amplitude of the pulmonary $\dot{V}O_2$ response, relative to placebo, was evident following NO_3^- supplementation during a step increment to the same absolute moderate-intensity cycling work rate. Accordingly, the functional 'gain' (i.e., the ratio of the increase in O_2 consumed per minute to the increase of external power output and the reciprocal of delta efficiency; 64) was reduced from 10.8 $mL \cdot min^{-1} \cdot W^{-1}$ following placebo supplementation to 8.6 $mL \cdot min^{-1} \cdot W^{-1}$ following NO_3^- supplementation. Moreover, the gross O_2 cost of exercise (comprising resting metabolic rate, the O_2 cost of moving the limbs during baseline pedaling, and the O_2 cost of muscle contraction to meet the imposed work rate) was reduced by ~5%. Importantly, the reduction in $\dot{V}O_2$, and thus of ATP re-synthesis through oxidative phosphorylation, was not compensated by elevations in glycolytic ATP provision, as inferred, albeit crudely, from the similar blood [lactate] values between the BR and placebo conditions. These findings extend those of Larsen et al. (45) by demonstrating that the O_2 cost of sub-maximal exercise is reduced following NO_3^- ingestion in the form of a natural food product. That heart rate (HR) and minute ventilation ($\dot{V}_E$) were not significantly different between treatments suggests that the reduction in $\dot{V}O_2$ originated from the skeletal muscles, and not from alterations in the energetic cost of cardio-respiratory support processes. Moreover, the similar RER between the conditions indicates that substrate utilization (which can influence the O_2 cost of exercise) was not altered by the intervention.

The mechanistic bases for the reduced O_2 cost of sub-maximal exercise following increased NO_3^- intake either by pharmacological (45) or natural dietary means (present study) are unclear. The inhibition of NO synthesis has previously been shown to increase steady-state $\dot{V}O_2$ in dogs (56) but not humans (38) or horses (41). It is widely accepted, however, that NO is involved in the regulation of mitochondrial O_2 consumption. In particular, it has been established that NO has a strong affinity for cytochrome c oxidase (CytOX; 10), but there is also evidence that NO has the potential to modulate other aspects of mitochondrial and muscle contractile function (11, 57, 58).

A reduction in the O_2 cost of mitochondrial ATP re-synthesis would require either more protons pumped per O_2 molecule reduced, or the use of an alternative terminal electron acceptor in place of O_2. It has been proposed that mitochondrial efficiency is intimately linked to the process of uncoupled respiration in which mitochondrial proton leak results in energy dissipation as heat instead of conversion to ATP (8). In this regard, the improved O_2 efficiency noted in the present study following BR ingestion might be related to a reduction of mitochondrial proton leak or proton pump slippage. There is evidence to suggest that NO increases the efficiency of oxidative phosphorylation in isolated mitochondria by reducing slipping of the proton pumps (17). Another possibility is that NO_2^- could be acting in place of O_2 as the final electron acceptor in the respiratory chain, thereby reducing the requirement for O_2 consumption (3). An intra-mitochondrial NO_2^- regeneration pathway would be critical in this scenario, given the limited NO_2^- concentration within the mitochondria. Under conditions of low electron flux, NO can inhibit CytOX by binding to the Cu^{2+} active site yielding nitrosonium (NO^+) which is subsequently hydrated to NO_2^- (18). One possible scenario is that the NO_2^- so produced could be reduced to NO by accepting an electron from CytOX, and this NO could subsequently bind to the Cu^{2+} active site, completing the cyclical process to regenerate NO_2^-. This possibility is intriguing as the hydration of NO^+ to NO_2^- yields an electron which can be redistributed within CytOX (19, 60). Subsequently, this electron may be accepted by NO_2^-, potentially utilizing the electron derived from its synthesis, and could contribute to proton pumping and ATP synthesis, in an efficiently-coupled process.

The reduction in O_2 consumption with increased dietary NO_3^- intake could also be attributed, in part, to a reduced ATP cost of force production, requiring less flux through oxidative phosphorylation. One of the most energetically costly processes during skeletal muscle contraction is sarcoplasmic reticulum (SR) Ca^{2+} pumping, which may account for up to 50% of the total ATP turnover (7). The presence of reactive oxygen species (ROS) increases the opening probability of the SR Ca^{2+} release channels (48), and the active reuptake of the elevated cytosolic Ca^{2+} would present a considerable energetic challenge (7). NO donors which invoke small elevations in NO might protect the channel against oxidation-induced Ca^{2+} release, without significantly altering channel function (53). Therefore, the BR-induced elevations in NO may have prevented an excess of Ca^{2+} release, and subsequently reduced the considerable energetic cost of its re-sequestration. These suggestions are naturally speculative at the present time and await further investigation.

Effects of dietary nitrate on the physiological responses to severe exercise

The physiological responses during severe exercise were different to those observed during moderate exercise, suggesting that the influence of NO_2^- and/or NO on muscle function are specific to the exercise intensity domain being investigated. Firstly, there were no significant differences in NIRS-derived indices of muscle oxygenation between the NO_3^- and placebo conditions during severe exercise, though this might be a function of the fact that severe exercise always followed moderate exercise in our experiments. Moreover, in contrast to the reduced steady-state $\dot{V}O_2$ observed during moderate exercise following increased NO_3^- consumption, the amplitude of the primary or fundamental component $\dot{V}O_2$ response during severe exercise was increased (by ~7%) and the amplitude of the subsequent $\dot{V}O_2$ slow component was reduced (by ~23%), with the $\dot{V}O_2$ at the point of task failure being not significantly different between the conditions. These changes in $\dot{V}O_2$ kinetics during high-intensity exercise following dietary NO_3^- supplementation resemble those which are observed following an initial 'priming' bout of high-intensity exercise (15, 35, 45), effects which have been attributed, either separately or in combination, to increased muscle O_2 delivery, increased oxidative metabolic enzyme activity and carbon substrate availability, and altered motor unit recruitment patterns (15, 35, 45). We have previously reported that NOS inhibition with L-NAME significantly increased the amplitude of the $\dot{V}O_2$ slow component and speculated that this might be related to changes in muscle O_2 delivery or its distribution and/or to (related) changes in motor unit recruitment patterns (39). It is presently unclear which, if any, of the above-named mechanisms contributed to the altered $\dot{V}O_2$ kinetics during severe exercise following dietary NO_3^- supplementation. Myocytes in close proximity to a capillary are advantaged with respect to muscle O_2 availability, whereas myocytes situated further away are increasingly less well supplied with O_2, creating an O_2 pressure gradient within the contracting muscles. The hypoxic and acidic milieu within and surrounding the distal myocytes might stimulate NO_2^- reduction to NO facilitating vasodilatation, and thus delivery of O_2. The NO so produced is capable of diffusion and may inhibit mitochondrial O_2 consumption in the myocytes proximal to the capillary bed, promoting deeper diffusion of the available O_2 (32, 59) and therefore enabling a more appropriate matching of local O_2 delivery to O_2 requirement. Another possibility is that the increased NO availability following increased dietary NO_3^- intake promotes mitochondrial biogenesis (16, 50). However, irrespective of the mechanism(s) involved, the return of $\dot{V}O_2$ kinetics *towards* first-order linear system dynamics during severe exercise will likely limit the rate at which metabolites which have been associated with the fatigue process (e.g., H^+, P_i, ADP) accumulate in skeletal muscle (14), effects which would be expected, in turn, to portend enhanced exercise tolerance.

The τ_p was significantly longer (i.e. phase II pulmonary $\dot{V}O_2$ kinetics was slower) following NO_3^- supplementation relative to placebo for severe exercise (group mean τ_p 40 *vs.* 33 s for BR and placebo, respectively). This is consistent with our previous reports that the τ_p was significantly faster when NOS activity was inhibited by L-NAME (38, 39, 66). The τ_p was also slightly longer during moderate exercise (group mean τ_p 29 *vs.* 26 s for BR and placebo, respectively), but this difference was not statistically significant. While it is acknowledged that changes in muscle blood flow, which might occur with greater or lesser NO availability, have the potential to dissociate the normally close relationship between muscle and pulmonary $\dot{V}O_2$ kinetics (2), our data suggest that NO might have an important regulatory influence on the inertia of $\dot{V}O_2$ dynamics following the onset of exercise (36, 51). It is of interest that NO_3^- supplementation resulted in changes in the amplitudes of the $\dot{V}O_2$ response to exercise (i.e., lower steady-state $\dot{V}O_2$ during moderate exercise and higher $\dot{V}O_2$ fundamental component and reduced $\dot{V}O_2$ slow component during severe exercise) while also slowing the phase II $\dot{V}O_2$ kinetics. While the mechanistic bases of this effect is not clear, it is possible that increased NO availability results, simultaneously, in a slowing of the rate at which $\dot{V}O_2$ rises following the onset of exercise (via competitive inhibition of cytochrome c oxidase; 10, 38, 39, 41, 56) and changes in the amplitudes of the fundamental and slow components of $\dot{V}O_2$ (through effects on the efficiency of muscle oxidative metabolism and/or contractile function; 3, 11, 17, 57).

The kinetics of $\dot{V}O_2$ is considered to be an important determinant of exercise tolerance (14, 36). However, in this study, we observed a 16% improvement in the time to task failure during severe exercise in the NO_3^- condition despite the slower phase II $\dot{V}O_2$ kinetics. Another parameter of $\dot{V}O_2$ dynamics considered to influence exercise tolerance is the slow component rise in $\dot{V}O_2$ observed during supra-GET exercise since this parameter is associated with greater utilization of the finite phosphocreatine (55) and glycogen (42) reserves. Indeed, a reduction in the $\dot{V}O_2$ slow component amplitude has been associated with improved exercise tolerance (1, 14). However, in the present study, the improvement in severe exercise tolerance with NO_3^- supplementation was not significantly correlated with the reduction of the $\dot{V}O_2$ slow component amplitude. The $\dot{V}O_2$ at task failure (which was equivalent to the pre-established $\dot{V}O_2$ max) was not different between conditions suggesting that the $\dot{V}O_2$ max was reached more slowly and/or could be sustained for longer following NO_3^-supplementation. It is noteworthy that the $\dot{V}O_2$ max during severe exercise was not impaired with NO_3^- supplementation although the steady-state $\dot{V}O_2$ was reduced during sub-maximal exercise. These results differ from those obtained with NOS inhibition. With the latter, $\dot{V}O_2$ max and exercise tolerance are impaired during both ramp incremental exercise (37) and supra-maximal step exercise (66). Although the mechanism for the enhanced performance observed in the present study is uncertain, an interesting possibility is that an elevation of tissue s-nitrosothiols with increased dietary nitrate intake (13) prevents thiols undergoing irreversible oxidative modification as a consequence of the production of ROS (12, 54), an effect which is known to compromise skeletal muscle force production (25). We wish to stress here that while the 16% improvement in the time to task failure during severe constant-work-rate exercise is impressive, the magnitude of effect would be expected to be much smaller during time-trial exercise tasks in which a given distance is completed in the shortest possible time (34). Nevertheless, it is possible that the effect might still be meaningful in terms of performance enhancement.

Applications and conclusions

A short period of dietary NO_3^- supplementation through a natural food product resulted in increased plasma [nitrite] and reduced systolic blood pressure in the normotensive young adult males who participated in our study. During exercise at a fixed moderate work rate, increased NO_3^- intake resulted in improvements in NIRS-derived indices of muscle oxygenation and a significant reduction in pulmonary $\dot{V}\text{o}_2$. It should be stressed that the remarkable reduction in the O_2 cost of sub-maximal exercise following dietary supplementation with inorganic nitrate in the form of a natural food product cannot be achieved by any other known means, including long-term endurance exercise training (1, 15, 49, 65). Although not directly tested in the present study, the results suggest that increased dietary NO_3^- intake has the potential to enhance exercise tolerance during longer-term endurance exercise. Moreover, in certain human populations (including the senescent and those with cardiovascular, respiratory or metabolic diseases), the activities of daily living are physically difficult because they have an energy requirement that represents a high fraction of the $\dot{V}\text{o}_2$ max. A reduction in the $\dot{V}\text{o}_2$ associated with such activities following dietary nitrate supplementation therefore has the potential to improve exercise tolerance and the quality of life in these groups. During exercise at a fixed severe work rate, BR ingestion reduced the amplitude of the $\dot{V}\text{o}_2$ slow component and increased the time to task failure by ~16%, suggesting that dietary nitrate supplementation might enhance high-intensity exercise performance. Further research is required to investigate the mechanistic bases for the reduced O_2 cost of sub-maximal exercise observed with increased dietary nitrate intake in this study and previously (45; i.e., reduced ATP cost of force production and/or increased mitochondrial P/O ratio). Finally, the possible ergogenicity of dietary nitrate supplementation during different types of exercise in humans is likely to be a fertile area for further research.

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